425

THE RENAL REGULATION OF THE EXTRACELLULAR IONIC PATTERN DURING HYPERVENTILATION

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The renal excretion of water and electrolytes appears to be modified by stimuli resulting from change in the volume, osmotic pressure, and ionic pattern of the extracellular fluid (Welt, 1952; Smith, 1952; Denton *et al.*, 1952a). The experiments to be reported in this paper are part of an investigation into the mechanisms of the renal component of regulation of the extracellular ionic pattern. Hyperventilation produces a fall in the partial pressure of CO_2 in the extracellular fluid. As a consequence the concentration of dissolved CO_2 and of carbonic acid falls. If the bicarbonate concentration remains constant, the extracellular pH rises, in accordance with the prediction of the Henderson-Hasselbalch equation for the carbonic acid-bicarbonate buffer: $pH = 6{\cdot}1 + \log \frac{\{BHCO_3\}}{\{HHCO_3\}}$. It is known that chronic hyperventilation in man, produced by acclimatization at high altitude, is associated with a low extracellular bicarbonate concentration and a high ratio of chloride to sodium (Dill, Talbott, and Consolazio, 1937). Experiments in controlled passive ventilation by Eichelberger and Hastings (1937) indicate that in the dog hyperventilation for a period of one hour produces similar changes in the extracellular fluid, although compensation is not complete in that the pH rises considerably. Studies on renal excretion during hyperventilation by McCance and Widdowson (1936) and Stanbury and Thomson (1952) show that in man voluntary overbreathing for a period of approximately 30 minutes produces an increase in bicarbonate excretion both absolutely and relatively to the two main urinary cations sodium and potassium, a decrease in chloride excretion relatively to sodium and potassium, and an increase in the urinary pH. An increase in urinary flow was observed by McCance and Widdowson, but not by Stanbury and Thomson. The latter authors found that, in the short period of their experiments, although a definite fall in the extracellular bicarbonate concentration occurred, there was no alteration in the concentrations of sodium and chloride. They concluded that an increase in the concentration of organic acids restored the extracellular anion-cation balance. They also observed a fall in the extracellular potassium and phosphate concentrations. The extracellular

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and urinary electrolyte patterns returned to baseline after the hyperventilation period. McCance and Widdowson, and Stanbury and Thomson found that "salt deficiency," produced by deficient NaCl intake over several days, considerably reduced, or abolished, the renal response referred to above. McCance and Widdowson showed that the renal response was related to the decreased pCO_2 and not to the muscular effort of overbreathing.

Collectively, these findings suggest that by reduction of the carbonic acid component, interference with the extracellular "bicarbonate buffer" produces a renal response of increased bicarbonate excretion and decreased chloride excretion relatively to total cation excretion, and that this response produces or maintains a lowered extracellular concentration of bicarbonate and an increased concentration of chloride without change in the sodium concentration. However, there is no direct evidence that this is the case, and the present experiments were designed so that the renal response to hyperventilation could be studied over a period of time sufficient for an alteration in the extracellular ionic pattern to occur.

EXPERIMENTAL METHODS.

The subjects were six healthy adult males between the ages of 20 and 30 years. Two of these were studied both during a period of hyperventilation and during a control experiment some weeks later. Prior to each experiment the subject fasted overnight, and had a light breakfast consisting of tea or milk and one or two slices of buttered toast, at least three hours before the commencement of the experiment. Water intake in this three-hour period was maintained at the rate of 200–300 ml. per hour. Water intake during the experiment was maintained by the ingestion of 50 ml. of tap water every ten minutes. Hyperventilation was induced in a conventional suction type respirator unit, at a rate of 30 per min., and a pressure fluctuation of 0 to —30 cm. of water. After a period of 30–60 minutes at rest on a water intake of 50 ml. every 10 minutes, baseline samples of blood and urine were obtained, the subject was weighed, and hyperventilation was started. During the period of hyperventilation, blood samples were taken from an antecubital vein through arm ports in the side of the respirator. Local infiltration of the skin and tissues adjacent to the vein with 5 p.c. procaine was performed before each venipuncture. The purpose of this was not only to minimize the pain of repeated venipuncture, but also to prevent spasm of the vein on insertion of the needle. Urinary samples were excreted into wide-mouthed Erlenmeyer flasks containing a one-inch layer of paraffin oil, and removed from the respirator *via* the arm ports. Water intake was provided through a rubber tube from a graduated cylinder, and glucose was provided according to individual requirements. After a period of hyperventilation lasting approximately four hours, a final sample of blood and urine was obtained, and passive hyperventilation was stopped. The subject was then re-weighed, and allowed to rest either reclining or seated. At least one blood sample was obtained in the post-hyperventilation period, which lasted approximately two hours. Half-hourly or hourly urine collections were made in this period, depending on the rate of urine flow.

The plan for the control experiments was exactly the same as for the hyperventilation experiments, except that the arm ports of the respirator were left open and the subject respired normally. The constricting collar about the neck was adjusted as for hyperventilation.

Chemical Methods.

The methods used for the determination of haematocrit, plasma protein, plasma and urinary Na, K, Cl, HCO_3, have been described elsewhere (Harris, McDonald and Williams, 1952; Denton, Maxwell, McDonald, Munro and Williams, 1952b).

Blood pH was determined at room temperature with a glass electrode and a Jones Model "B" pH meter. The reading was corrected to room temperature by the formula $pH_{38} = pHt - 0{\cdot}0147\ (38-t)$ (Rosenthal, 1948). No temperature correction was used for the urinary pH.

Plasma pCO_2 was calculated from the blood pH and plasma bicarbonate values, using the nomogram of Singer and Hastings (1948).

Urinary ammonia was determined by alkalinization of the urine and steam distillation of the ammonia into boric acid. The ammonia was then titrated directly with standard alkali.

Plasma and urinary creatinine was estimated colorimetrically by the method of Folin and Wu (1919).

RESULTS.

Effect of hyperventilation on pCO_2, pH and ionic pattern of the extracellular fluid.

Table 1 shows clearly that the technique employed was effective in lowering plasma pCO_2 and raising the blood pH, with one exception (J.B-W.). The average greatest fall in pCO_2 was 20 mm. Hg., and the average greatest rise

TABLE 1.

	Time mins.	pCO_2 mm.Hg.	Blood pH	Plasma HCO_3 mm./l.	Plasma Cl/Na
I.R.M.	−15	38	7·44	25·4	0·736
	0				
	+60	—	7·50	—	—
	+120	—	7·34	—	—
	+160	44	7·35	24·5	0·779
	+200	65	7·24	27·6	0·745
	+250	50	7·32	26·7	0·726
W.B.M.	−12	53	7·30	26·3	0·723
	0				
	+34	54	7·29	24·4	0·731
	+75	45	7·36	24·0	0·753
	+130	35	7·40	21·3	0·733
	+175	37	7·41	22·6	0·769
	+198	44	7·33	23·0	0·773
	+250	63	7·20	24·3	0·764
J.M.	−17	64	7·26	27·6	0·78
	0				
	+28	48	7·34	26·0	0·80
	+69	52	7·30	25·3	0·82
	+129	38	7·43	25·3	0·84
	+172	36	7·46	25·4	0·80
	+211	29	7·50	21·9	0·81
	+227	32	7·45	21·9	0·81
	+269	69	7·21	27·9	0·82
	+301	63	7·24	27·0	0·80
J.B-W.	−10	39	7·42	25·5	0·714
	0				
	+28	35	7·45	24·2	0·730
	+85	39	7·41	24·7	0·750
	+240	45	7·40	26·8	0·750
	+285	—	—	26·8	0·730
D.K.J.	−18	52	7·34	26·6	0·762
	0				
	+30	35	7·42	23·4	0·754
	+69	36	7·40	23·4	0·741
	+107	38	7·42	23·6	0·770
	+153	37	7·37	21·4	0·787
	+194	37	7·37	21·2	0·759
	+248	43	7·37	24·2	0·764
	+326	52	7·32	26·4	0·743
E.M.H.	−9	59	7·28	26·7	0·722
	0				
	+27	37	7·37	21·0	0·775
	+59	33	7·42	21·4	0·789
	+91	32	7·46	22·4	0·795
	+129	30	7·43	20·5	0·808
	+168	35	7·37	19·9	0·794
	+237	42	7·34	22·0	0·787
	+281	47	7·34	25·4	0·743
	+326	50	7·32	25·5	0·756

in blood pH was 0·13 pH units. Associated with these changes, but not commensurately related in time, was a fall in the plasma HCO_3^- concentration and a rise in the Cl^- concentration, both absolutely and relatively to the Na^+ concentration. The Na^+ concentration fluctuated ±4–6 m.eq./l. in individual subjects (Fig. 1), and although in one instance (W.B.M.) there was an initial rise, the trend was for the Na^+ concentration to fall slightly, augmenting the rise in the plasma Cl:Na ratio. In addition to these changes in the main ionic constituents of the extracellular fluid there was a fall in the K^+ concentration, the mean fall being 0·9 m.eq. per litre. In four experiments of six, the fall in extracellular K^+ concentration continued after hyperventilation was stopped. In all experiments the blood pH and the ratio Cl/Na returned to or near baseline after hyperventilation, although in three the blood pH first fell below the baseline figure.

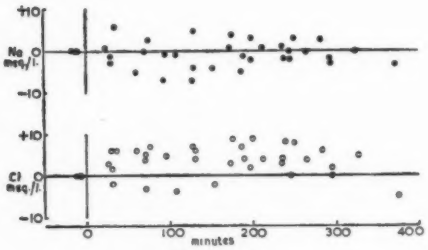


Fig. 1 (a).

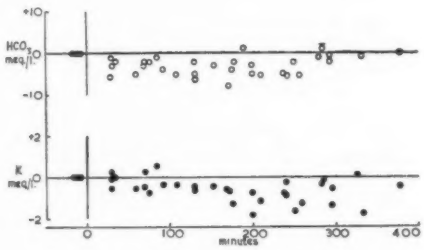


Fig. 1 (b).

Fig. 1 (a). Change in Na^+ and Cl^- concentrations in blood plasma.
Mean concentrations before hyperventilation: $Na^+ = 140$, $Cl^- = 104$.
Mean concentrations at end of hyperventilation: $Na^+ = 140$, $Cl^- = 109$.

Fig. 1 (b). Change in plasma HCO_3^- and K^+.

The degree of the changes described above varied in individuals, and appeared to be related to the efficiency of passive respiration. It is fairly easy for a normal individual to resist the action of these respirators, and although the subjects were well aware of the purpose of the experiment, co-operation was impaired by the onset of drowsiness and euphoria a short time after hyperventilation was started. In addition, tingling sensations and mild tetany occurred to a variable degree. In one instance (J.B-W.) the absence of any marked rise in blood pH or fall in plasma pCO_2 is assumed to be due to ineffective respiration due to the above factors. The renal response to hyperventilation in this subject was slight, although there was evidence of changes in distribution of water, Na^+ and HCO_3^- during attempted hyperventilation.

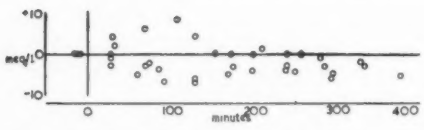


Fig. 1 (c). Change in "undetermined" plasma anion concentration.

Renal excretion of water and electrolytes.

There was a striking increase in the rates of excretion of all the measured urinary constituents except HCO_3^- in the first 30–60 minutes after hyperventilation was started (Figs. 2 and 3). This increase in urinary output was not sustained, but was followed by a reduction, which occurred more rapidly in some experiments than in others. In two experiments (W.B.M. and J.M.) an initial fall in water and electrolyte excretion rates occurred. These subjects had a high rate of urinary flow before hyperventilation was started. In those experiments in which the urinary output fell rapidly after the initial rise, a second rise occurred during the hyperventilation period, to be succeeded by a second fall, which persisted after hyperventilation was stopped. The initial rise in urinary output is in accordance with the findings of McCance and Widdowson (1936). In the experiments by Stanbury and Thomson (1952) a similar rise is described in only one subject.

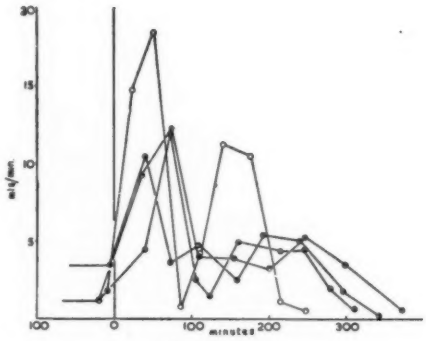


Fig. 2 (a).

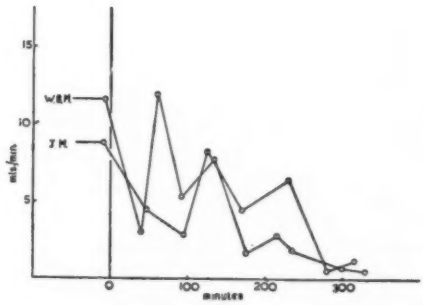


Fig. 2 (b).

Fig. 2 (a). Rate of excretion of water in subjects with initial rate of less than 5 ml./min.

Fig. 2 (b). Rate of excretion of water in subjects with initial rate of more than 5 ml./min.

The urinary pH rose during hyperventilation to between 7·2 and 7·9, the degree of rise being greatest in those subjects with an initial pH of 6 or less (Fig. 3). The least rise in pH was found in J.B-W. with whom, as already discussed, the respirator action was least effective.

The renal excretion of bicarbonate, chloride, sodium and potassium is best described by considering changes in the anion : cation ratios in the urine. A reciprocal change occurred in the ratios Cl/Na+K and HCO_3/Na+K, the former falling and the latter rising during the period of hyperventilation (Fig. 5). The changes in these ratios are related to the lowered pCO_2 and raised blood pH of the extracellular fluid and not to the diuresis which occurred. They were

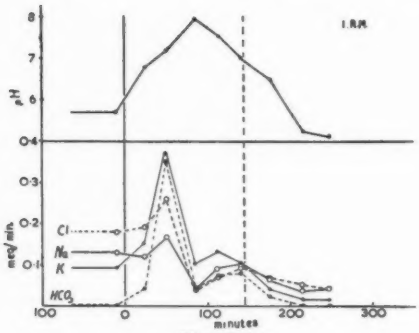


Fig. 3 (a).

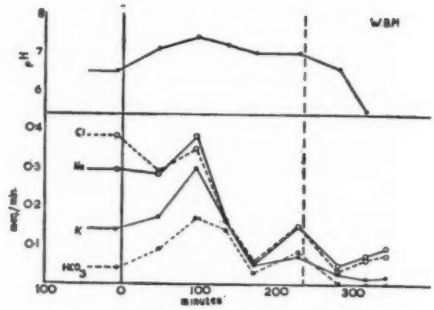


Fig. 3 (b).

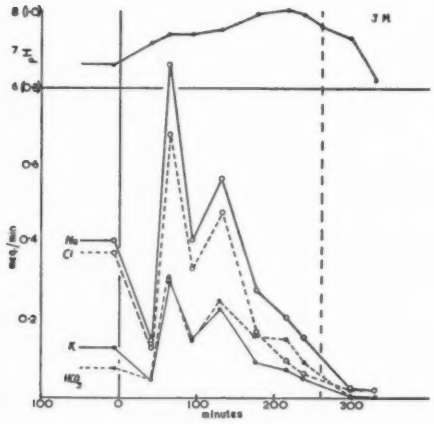


Fig. 3 (c).

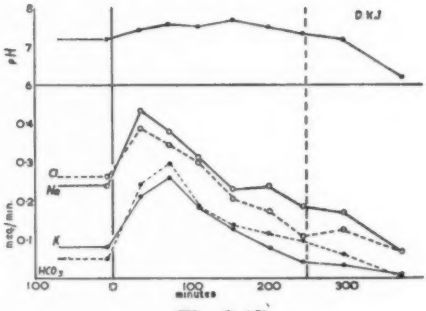


Fig. 3 (d).

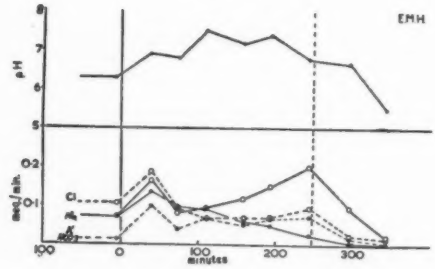


Fig. 3 (e).

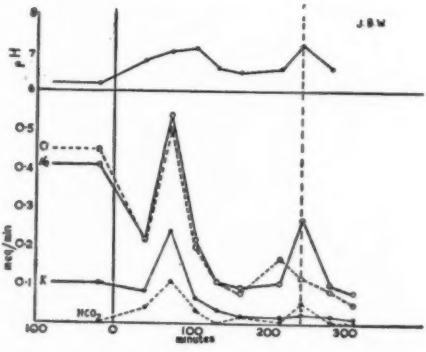


Fig. 3 (f).

Fig. 3 (a, b, c, d, e, f). Urinary pH and rate of excretion of Na^+, K^+, Cl^- and HCO_3^-.

achieved by an increase in HCO_3^- excretion relatively to Na^+, K^+ and Cl^-, and by an increase in the excretion of Na^+ and K^+ relatively to Cl^-. After hyperventilation was stopped, the return of these ratios towards the baseline values was the result of a decrease in HCO_3^- excretion relatively to Na^+ and K^+ and Cl^- excretion, and an increase in Cl^- excretion relatively to Na^+ and K^+. These post-hyperventilation changes were not associated with any consistent changes in urinary water excretion, but were related to the increase in plasma pCO_2 and fall in blood pH.

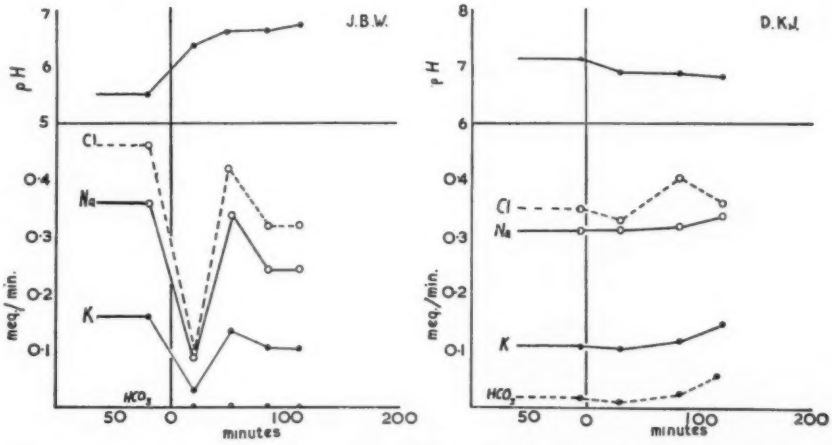


Fig. 4. Urinary pH and rate of excretion of Na^+, K^+, Cl^- and HCO_3^- in control subjects.

Correlation of K^+ excretion with N_2 excretion shows that in each experiment a rapid increase in K^+ excretion above that theoretically due to cellular catabolism (2·3 m.eq. per gm. of urinary N_2) occurred shortly after hyperventilation was started. After hyperventilation was stopped, the $K:N_2$ ratio fell to or below the fasting figure of 2·3:1 (Gamble, Ross and Tisdall, 1923). Although the subjects commenced the experiment in a semi-fasting state, the urinary $K:N_2$ ratio in the pre-hyperventilation period was always greater than 2·3:1. However, this "excess" K^+ was obviously "cleared" by the end of the experiment (Fig. 6). Analysis of the K^+ balance over the hyperventilation period indicates that the fall in extracellular K^+ concentration could be accounted for on the basis of renal excretion of K^+ in excess of that due to metabolic breakdown. The fact that the urinary $K:N_2$ ratios in the two control subjects were consistently above 2·3:1, and rose slightly towards the end of the control period, suggests that the fall of the $K:N_2$ ratio to or below 2·3:1 in the post-ventilation period was due to active retention of K^+ as a result of a body deficit.

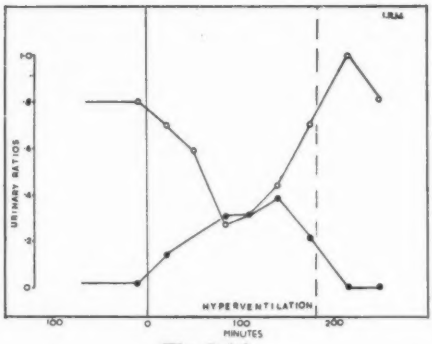
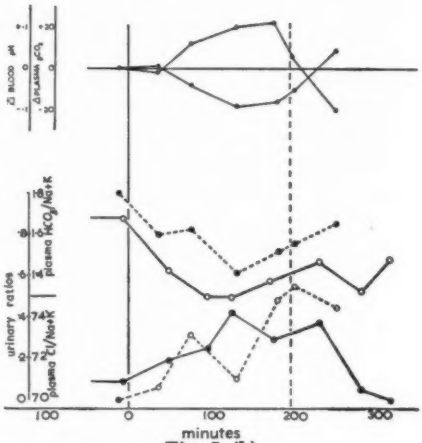


Fig. 5 (a).



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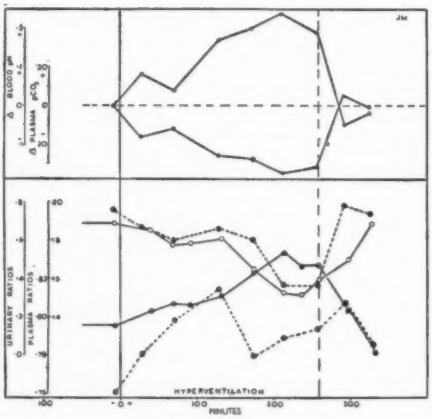

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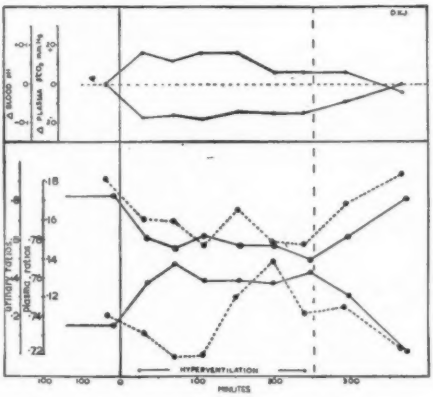

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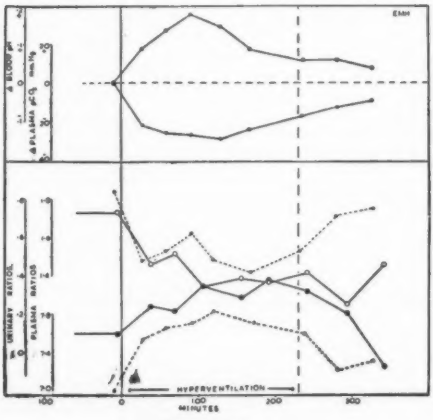

Fig. 5 (e).

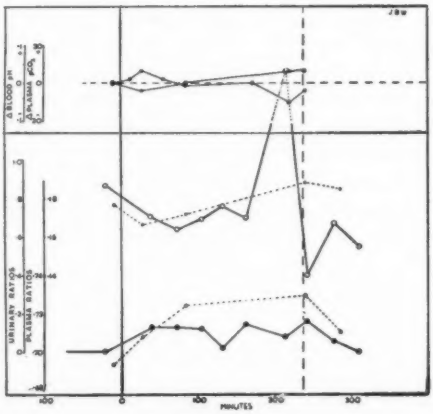

Fig. 5 (f).

The urinary NH_3 excretion fell immediately after hyperventilation was started, and remained low during the period of hyperventilation. After hyperventilation was stopped, the urinary NH_3 excretion rose. These changes can be correlated also with the changes in the urinary pH (Fig. 7).

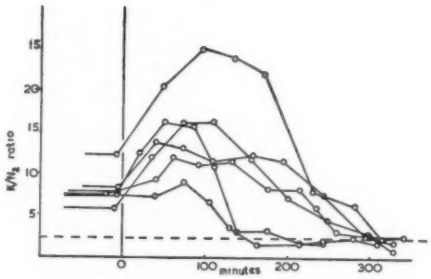

Fig. 6. Urinary K:N_2 ratios.

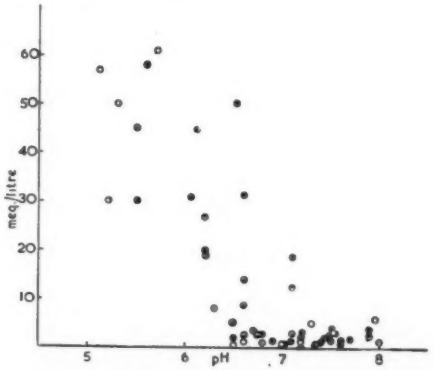

Fig. 7. Correlation between urinary NH_3 concentration and urinary pH.

Changes in the diffusion space of Na^+, K^+ and Cl^- (Table 2).

The weight loss and measured water balance during the hyperventilation period show an average water deficit of near one litre, the respiratory loss accounting for about two-thirds of the deficit. The change in diffusion space of Na^+ and Cl^- is calculated from an assumption of initial extracellular fluid volume of 16 p.c. of initial body weight. If the further assumption is made that Cl^- remains in the extracellular space, then the Cl^- balance indicates that a decrease in extracellular volume of 0·4 to 1·5 litres occurred. Using this conclusion, the Na^+ and HCO_3^- balances indicate a shift of Na^+ and HCO_3^- into the intracellular fluid, in a ratio of approximately $Na : HCO_3 = 2{\cdot}3 : 1$; and either a small or no shift of K^+ out of cells. This finding with regard to potassium is not in agreement with the proposition of Stanbury and Thomson (1952) that there is a shift of K^+ into cells during hyperventilation. The decrease in Cl^- space is in accordance with the findings of Eichelberger and Hastings (1937), who demonstrated by muscle studies a rise in the intracellular, and a fall in the extracellular, components of muscle water in respiratory alkalosis.

EXPLANATION OF FIG. 5.

Fig. 5 (a, b, c, d, e, f). Blood pH, plasma pCO_2 with urinary and plasma Cl/cation and HCO_3/cation ratios.

Urinary excretion: The points plotted represent the end of each collection period. The horizontal line joining the first point represents the duration of the first collection period.

The following conventions are used: urine—solid lines; plasma—dotted lines; ratio $HCO_3/Na+K$—solid circles; ratio $Cl/Na+K$—open circles.

Considering the fairly large errors inherent in the methods of calculation of changes in extracellular volume by this method, the fall in this volume agrees fairly well with the net water loss in each subject.

TABLE 2.

Balance data during period of hyperventilation.

		External Balance	E.C.	I.C.
I.R.M.	H_2O ml.	−1,000	−1,200	+200
		Resp. Loss ?		
	Na m.eq.	−17	−241	+224
	K ,,	−26	−17	−9
	Cl ,,	−23	−23	0
	HCO_3 ,,	−8	−44	+36
W.B.M.	H_2O ml.	−800	−1,500	+700
		Resp. Loss 600		
	Na m.eq.	−41	−163	+122
	K ,,	−30	−30	0
	Cl ,,	−38	−38	0
	HCO_3 ,,	−23	−77	+54
J.M.	H_2O ml.	−800	−800	0
		Resp. Loss 505		
	Na m.eq.	−82	−182	+100
	K ,,	−30	−17	−13
	Cl ,,	−61	−61	0
	HCO_3 ,,	−38	−92	+54
J.B-W.	H_2O ml.	−700	−800	+100
		Resp. Loss 165		
	Na m.eq.	−56	−122	+66
	K ,,	−19	−10	−9
	Cl ,,	−53	−53	0
	HCO_3 ,,	−10	−48	+38
D.K.J.	H_2O ml.	−1,000	−400	−700
		Resp. Loss 365		
	Na m.eq.	−74	−98	+24
	K ,,	−32	−7	−25
	Cl ,,	−62	−62	0
	HCO_3 ,,	−45	−70	+25
E.M.H.	H_2O ml.	−800	−900	+100
		Resp. Loss 410		
	Na m.eq.	−37	−113	+76
	K ,,	−18	−6	−12
	Cl ,,	−23	−23	0
	HCO_3 ,,	−19	−55	+36

Changes in endogenous creatinine clearance.

Endogenous creatinine clearances on two subjects during hyperventilation increased during the diuresis, then decreased to near the pre-hyperventilation figure and rose again slowly for the remainder of the hyperventilation period (Fig. 8). Inulin clearances fell during hyperventilation in Stanbury and Thomson's experiments. It may be that the initial high water loads in the latter experiments were responsible for the different clearance findings, although there are insufficient data for valid comparison.

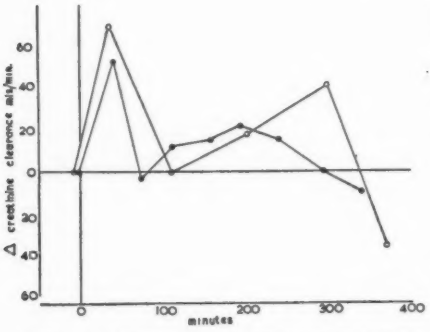

Fig. 8. Change in endogenous creatinine clearance.

Open circles: D.K.J.
Solid circles: E.M.H.

DISCUSSION.

The purpose of these experiments was to determine the significance of renal excretion of water and electrolytes in relation to the extracellular ionic pattern when the extracellular pH was raised. The change in ionic pattern which followed the lowering

of pCO_2 confirms the findings of Eichelberger and Hastings, and of Dill *et al.* The alteration in the excretion pattern of Na^+, K^+, Cl^- and HCO_3^- confirms the findings of McCance and Widdowson, and of Stanbury and Thomson, and the longer time period of the experiments enables one to relate these urinary changes to the extracellular changes. The increase in urinary volume confirms the findings of McCance and Widdowson, and differs from those of Stanbury and Thomson. The absence of any increase in urinary volume in the latter experiments was almost certainly related to the abnormally high initial rates of urinary excretion induced by ingestion of water during the pre-hyperventilation period. It should be noted that although there was an initial high rate of urinary flow in their experiments, the excretions of Na^+, K^+, Cl^- and HCO_3^- showed the same rise immediately after hyperventilation as in the present experiments and in those of McCance and Widdowson. As the initial urinary excretion rates were in the normal range in the present experiments, it is concluded that increased excretion of water is part of the renal response.

It has been suggested earlier (Denton *et al.*, 1952a, 1952b) that a constancy displayed in the ratio Cl : Na in extracellular fluid has some biological significance. The ratio was shown to be constant in circumstances where sodium was lost from the body in excess of chloride, the constancy being maintained by a differential renal excretion of these two ions. The fact that this ratio is remarkably constant throughout the animal kingdom was cited in support of this proposition. In the present experiments, it cannot be said that the change in the renal excretion of electrolytes was such as to correct the change in extracellular Cl : Na ratio, nor to maintain a normal bicarbonate concentration as is suggested by Pitts (1950). The increased bicarbonate excretion and fall in the urinary Cl : Na ratio would rather augment these changes. The experimental data show a close correlation between the plasma HCO_3/NA+K ratio, and the urinary Cl/Na+K ratio, which indicates that the renal conservation of Cl relative to cation is at least part of the mechanism producing the plasma change (Fig. 5). In general, a similar correlation between plasma Cl/Na+K and urinary HCO_3/Na+K is evident. Occasional divergence between the latter two ratios is due to fluctuations in the small unmeasured anion concentration in plasma[2]. The fact that fluctuations in the plasma ratio Cl/Na+K are not reflected in renal excretion is evidence that (a) the true correlation is between the reciprocal of the plasma ratio HCO_3/Na+K and the urinary ratio HCO_3/Na+K, and (b) the plasma ratio Cl/Na+K or, as previously postulated, Cl/Na does not *per se* influence renal excretory behaviour during hyperventilation.

[2] Two possibilities may account for this fluctuation: (1) inaccuracy in the plasma Cl estimation, which cannot be considered to be more accurate than ± 2 p.c.; (2) fluctuating organic anion concentrations. Because of the shape of the plasma Cl/Na curves, it is unlikely that technical error occurring in a consecutive series of estimations can account for the changes (e.g. J. M., Fig. 5 (c), points 5, 6 and 7).

"Electrical neutrality" of the extracellular fluid requires either that some anion "replace" the reduced bicarbonate, or that the concentration of cation be reduced. In the voluntary hyperventilation studies of Stanbury and Thomson, the bicarbonate was "replaced" by an unidentified anion, presumably lactate. As the experiments must have involved a moderate degree of muscular work, this is a reasonable finding, and accounts for the absence of change in the extracellular Cl : Na+K ratio. In the present experiments the bicarbonate was "replaced" to a variable degree by an increase of the chloride concentration. It is significant that the renal excretion of electrolytes in both types of experiment was the same, notwithstanding the different plasma ionic patterns.

It must therefore be concluded that under these experimental conditions, the kidney did not respond to nullify the change in extracellular Cl : Na ratio, but in fact operated to augment it. A further conclusion is reached that the renal response of increased Na^+ and HCO_3^- excretion and relatively decreased Cl^- excretion, was in the direction of restoring the normal $\frac{BHCO_3}{HHCO_3}$ ratio, and so restoring pH to normal.

Further consideration of the data shows that the response of the organism to a change in the pCO^2 of the alveolar air has at least two components, a redistribution of water and electrolytes between intracellular and extracellular fluid, and differential renal excretion of water and electrolytes. The effect of this total response is to restore the extracellular pH towards its normal value by reducing the bicarbonate component of the carbonic acid-bicarbonate buffer pair. The studies by Dill *et al.* at high altitudes indicated that this response was not wholly effective over their period of study in that there was a definite progressive increase in blood pH with increase in altitude, although the blood pH of residents at high altitude was in the normal range. It is reasonable at this point to speculate that in the former case the altered distribution of water and electrolytes in the body fluids may establish a new equilibrium in which the extracellular pH is set at a higher value. It should be remembered that considerations of redistribution of water and electrolytes may apply with equal force to renal tubular cells as to muscle cells and that alterations in these cells may condition an altered renal response with a consequent reset equilibrium state. It is also possible, in view of Dill's findings on residents at high altitude, that once the redistribution occurs while the plasma pCO_2 remains low, correction (i.e. complete acclimatization) may take a considerable period of time. The findings of Gamble (1951) on the time required for the correction of the disturbed body fluid equilibrium following a single dose of $NaHCO_3$, favour this possibility.

The mechanism of the probable changes in volume of the extracellular compartment and movement of sodium and bicarbonate can only be a matter of speculation. However, it is known that loss of potassium from the body is

associated with a movement of sodium into the intracellular fluid (Gamble, 1951), and it is possible that the potassium deficit and redistribution of sodium in hyperventilation are related to a common mechanism.

The mechanism of the renal response has been discussed by Stanbury and Thomson (1952) in some detail, in relation to the tubular ion-exchange hypothesis of Berliner (1951). The present experiments establish that the stimulus giving rise to the renal response is either change in pH or in pCO_2 of the extracellular fluid. Stanbury and Thomson cite unpublished data which indicate that acute alkalosis induced by sodium bicarbonate administration produces a similar type of renal response, and conclude therefore that it is the change in extracellular pH and not pCO_2 or the concentration of bicarbonate which modifies the renal ionic excretion. Certain features of the renal excretion in the present experiments deserve consideration in relation to the possible mechanism. Perhaps the most striking feature is the fluctuation in the excretion rates of water and electrolytes. This fluctuation bears no relationship to the urinary pH (Fig. 3). Berliner postulates that when alkalosis is produced, the altered renal tubular behaviour to Na^+ and K^+ is due to decreased secretion of H^+ ion. As there is less H^+ ion available for exchange with Na^+, tubular reabsorption of Na^+ is achieved by exchange of Na^+ for K^+ secreted by the renal tubule. When carbonic anhydrase is inhibited by 6063, he finds that the urinary pH increases and that there is an increased excretion of water, Na^+, K^+ and HCO_3^-, which is similar to what is found in the early phase of the present experiments. However, in the present experiments, when the stress is maintained, the excretion rates of these ions fall, and then fluctuate in an irregular manner, but in a general way towards baseline. This cannot be explained by postulating a prolonged inhibition of carbonic anhydrase alone. Some other factor must be operating, and in view of the consistent finding of a reduction in extracellular Cl^- space, it is not unreasonable to correlate the diuresis followed by restabilization of urinary flow with this new body fluid equilibrium. It is possible that circulatory factors are directly responsible for the change in urinary excretion rates. Other investigators (Dripps, 1947; Clarke, 1952) have reported circulatory disturbances during hyperventilation. The endogenous creatinine clearances in the present experiments, although not wholly reliable as indices of glomerular filtration, suggest that rapid fluctuations in glomerular filtration rate occur. The work of Selkurt (1951) indicates that changes in renal haemodynamics are associated with changes in electrolyte excretion rates. In summary, if it be postulated that the renal excretory "load" is not constant during hyperventilation, then the qualitative nature of the renal response is consistent with Berliner's ion exchange hypothesis.

It is useful to compare the hyperventilation data with previously published findings on acute respiratory acidosis in sheep (Denton *et al.*, 1952a). These authors found that when the extracellular pCO_2 was raised, the blood pH fell,

the extracellular bicarbonate concentration rose, and the chloride concentration fell both absolutely and relatively to the Na^+ (or total cation) concentration. These changes were accompanied by a fall in the excretion rates of Na^+, K^+, Cl^- and HCO_3^-. In general there was a rise in the urinary Cl/Na+K ratio, and a fall in the HCO_3/Na+K ratio, although there was no clear-cut reciprocal relationship between the two. There was also evidence of a redistribution of water and electrolytes between the intracellular and extracellular compartments. The urinary findings of diminished Na^+ and K^+ excretion during acidosis are consistent with the ion-exchange hypothesis, but again there is evidence of some alteration in glomerular filtration, and it is possible that other factors related to a change in body fluid equilibrium were operating in addition.

When acidosis or alkalosis is produced by the subtraction of Na^+ or Cl^- from the body, the renal response is different from that observed when the stress on pH is produced by interference with respiration (Denton *et al.*, 1952a, 1952b). This difference may be due to the introduction in subtraction stresses of stimuli related to change in extracellular osmotic pressure and/or volume, which modify the renal response. The experiments of McCance and Widdowson, and of Stanbury and Thomson, indicate that preliminary sodium depletion greatly diminishes the usual renal response to hyperventilation. These findings are suggestive in relation to interfering mechanisms, and further experiments along these lines, for example to establish the level at which Na^+ depletion interferes, are warranted.

SUMMARY.

Hyperventilation in human subjects was produced by passive respiration for a period of three to four hours.

This caused the blood pH to rise, the extracellular bicarbonate concentration to fall, and the extracellular chloride concentration to rise both absolutely and in relation to the total cation concentration.

The urinary pH rose and the renal excretion of Na^+, K^+, Cl^- and HCO_3^- altered so that the ratio Cl/Na+K fell and the ratio HCO_3/Na+K rose. These changes were related to the change in blood pH (or pCO_2) and were such as would restore the extracellular $H_2CO_3 : BHCO_3$ ratio to normal. There was no correlation between changes in renal excretion rates of water and individual electrolytes and the blood pH (or pCO_2).

A redistribution of water and electrolytes occurred in the body fluids.

These findings are discussed in the light of previous experimental data, and in relation to the tubular ion-exchange hypothesis.

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SALMONELLA BACTERIOPHAGES

I. BACTERIOPHAGES OF *S. ADELAIDE*

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Atkinson *et al.* (1952) reported the occurrence of 12 lysogenic strains of *S. adelaide* among 48 tested. Ten of these lysogenic strains yielded phages showing two plaque types C (clear) and H (hazy). The other two lysogenic strains yielded phages with similar plaques different from either the C or the H plaque. The present paper gives some general characteristics of these phages, allocating them to two types, and also presents a scheme of grouping of strains of *S. adelaide* on lysis pattern or lysogenicity.

METHODS.

Isolation of phages. Four-hour broth cultures of the non-lysogenic indicator strain 14 and the lysogenic culture under investigation were inoculated together on the surface of a nutrient agar plate. Some of the mixed growth, which showed evidence of lysis, was emulsified in broth and centrifuged. The clear supernatant, which contained phage, was (1) serially transferred in broth several times with strain 14 or (2) plated out on agar with strain 14. The final broth supernatant from (1) was heated at 56° C. for 30 minutes and phages isolated by plating out with strain 14 in suitable dilution to give isolated plaques. Representatives of all plaque types present were picked off and further purified by single plaque selection until successive platings showed only one type of plaque. The final preparations were heated at 56° C. for 30 minutes to ensure bacteriological sterility. The plate from (2) containing well separated plaques was also used to isolate and purify the phages by the same treatment used in (1).

Titration of purified phages. The method was essentially that used by Craigie and Yen (1938) for typhoid Vi phages. Several tenfold dilutions of the phage preparation were tested on strain 14 and the titre or C.T.D. was the highest dilution producing confluent lysis. Plaque and patch characters were described from these tests.

Test for sensitivity to phage lysis. Here also the method was essentially that used for Vi phage typing of typhoid cultures by Craigie and Yen (1938). The phage was tested on the appropriate strain at the C.T.D. or other suitable dilution.

Heat resistance tests. One ml. of a suitable dilution of the phage in broth at pH 7 was placed in test tubes and held at the required temperature for 30 minutes. 0·5 ml. of the heated preparation was mixed with 0·5 ml. of a 4-hour broth culture of strain 14 in 10 ml. of nutrient agar. The same amount of unheated control phage preparation was treated in the same way. The plaque counts on the test and control plates were compared. All tests were made in duplicate.

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Preparation of phage antiserum. A phage preparation with C.T.D. at least 10^{-1} was injected into rabbits intravenously. Four injections, the first consisting of 1 ml. and the others of 2 ml. each, were given over 7 days and blood was collected 8–10 days later.

Titration of phage antiserum. The method followed that given by Burnet *et al.* (1937). Dilutions of antiserum were prepared and mixed in equal quantities with the homologous phage suitably diluted to give a countable number of plaques in the control. The phage-serum mixtures were held at 48° C. for one hour and then plated out in duplicate in agar with strain 14. A control consisting of phage dilution and diluted normal rabbit serum was treated in exactly the same way as the test. The number of plaques was counted on the test and control plates and the end titre of the serum was taken as the highest dilution completely inhibiting plaque formation.

Cross neutralization tests. The highest dilution of phage antiserum producing complete neutralization of homologous phage was mixed with an equal volume of the phage to be tested. Before mixing, the phage was suitably diluted to give a countable number of plaques in the control which consisted of equal volumes of phage dilution and normal rabbit serum diluted to the same extent as the antiserum. To check the potency of the antiserum a neutralization test with homologous phage was included where necessary. The serum-phage mixtures were held at 48° C. for one hour and then plated out in duplicate on strain 14. The number of plaques developing on each plate was counted. Complete neutralization was indicated by absence of plaques.

Production of resistant strains and lysogenicity. The resistant strains were produced from the indicator strain 14 by phage treatment. A four-hour broth culture was inoculated on to nutrient agar and the phage spotted on. The plate was incubated overnight at 37° C. and some of the resulting secondary growth in the phage spot was then scraped off and the same treatment repeated with a four-hour broth culture of this growth. This procedure was repeated up to six times. Finally the broth culture from the secondary growth was plated out on agar and separate colonies inoculated into broth, incubated for four hours at 37° C. and tested with representative phages including the phage used in treatment. Those showing resistance to phage action were tested for lysogenicity by cross-plating with strain 14.

RESULTS.

Isolation and range of action of phages. Of the twelve lysogenic strains (2, 13, 15, 16, 20, 37, 39, 40, 43, 45, 47 and 49) described by Atkinson *et al.* (1952) phages were isolated from strains 2 and 20 which produced similar plaques, and from strains 16, 37, 39, 40, 45 and 49 representative of the group producing the two plaque types, clear (C) and hazy (H). The phages thus obtained were labelled Ad2, Ad20, Ad16C, Ad16H, Ad37C, Ad37H, Ad39C, Ad39H, Ad40C, Ad40H, Ad45C, Ad45H, Ad49C and Ad49H. The results of testing these phages on the strains from which they were derived are given in Table 1.

These results confirmed those already reported for the lysogenic strains with a smaller range of phages (Atkinson *et al.*, 1952) and showed that the C and H phages, irrespective of strain of origin, lysed strains 2 and 20 but not strains 16, 37, 39, 40, 45 and 49 which provided C and H phages. Phage Ad2 or Ad20 lysed all strains except 2 or 20 respectively.

TABLE 1.

Reactions of purified phages with the strains from which they were derived.

Phage No.	Strain tested 2	16	20	37	39	40	45	49	14 (indicator)
Ad2	—	+	+	+	+	+	+	+	+
Ad16C	+	—	+	—	—	—	—	—	+
Ad16H	+	—	+	—	—	—	—	—	+
Ad20	+	+	—	+	+	+	+	+	+
Ad37C	+	—	+	—	—	—	—	—	+
Ad37H	+	—	+	—	—	—	—	—	+
Ad39C	+	—	+	—	—	—	—	—	+
Ad39H	+	—	+	—	—	—	—	—	+
Ad40C	+	—	+	—	—	—	—	—	+
Ad40H	+	—	+	—	—	—	—	—	+
Ad45C	+	—	+	—	—	—	—	—	+
Ad45H	+	—	+	—	—	—	—	—	+
Ad49C	+	—	+	—	—	—	—	—	+
Ad49H	+	—	+	—	—	—	—	—	+
Ad2 mutant	+	+	+	+	+	+	+	+	+
Ad20 mutant	+	+	+	+	+	+	+	+	+

+ = lysis. — = no lysis. Phage preparation used undiluted.

When the lysogenic strains producing the C and H phages were investigated by method 1 which included preliminary serial transfer in broth, both C and H types of plaque appeared on plating, the H plaque predominating. However, when method 2 was used for isolation of phage from the lysogenic strains only H type plaques were found. These results were obtained with strains 16, 37, 39, 40, 45 and 49. Strain 37 was more intensively investigated for presence of C phage by picking numerous single colonies and investigating each by method 2 for type of plaque produced. Only H plaques were found. Apparently the C phages were not present in the lysogenic strains. The origin of the C phage was investigated by serially propagating the purified phages Ad16H, Ad37H and Ad49H which had been prepared from single H plaques from a succession of platings. After four to eight transfers in broth the phages were titrated and all yielded some C type plaques in the lower dilutions. In the upper dilutions the number of C type plaques rapidly decreased and none was observed in the highest dilutions showing numerous H type plaques. Thus the C phages arose from the H phages and were not present in the lysogenic strains. They were considered to be plaque-type mutants of the H phages.

Phages Ad2 and Ad20 showed no obvious plaque-type mutants on serial propagation although the plaques became clearer. However, they produced host-range mutants acting upon the strain from which the phage originally came. Both Ad2 and Ad20 when first isolated and purified showed the reactions given in Table 1. However, after serial propagation undiluted preparations of both phages lysed the parent strain, which was not affected by higher dilutions from which single plaques provided phage no longer acting on the parent strain.

By plating on the parent strain Ad2 and Ad20 were obtained as single plaques which yielded phage acting on the parent strain and thus on all strains of *S. adelaide* included in Table 1. These phages are recorded in Table 1 as Ad2 mutant and Ad20 mutant. Further work on plaque-type and host-type mutants of *S. adelaide* phages is proceeding and will be reported separately.

Plaque characters. The plaques produced by Ad2 and Ad20 were similar. They were small with rather irregular edge, tending to form satellite plaques and spread. On first isolation they showed much secondary growth but later became clearer with central spot of secondary growth. The plaques of Ad16C, Ad37C, Ad39C, Ad40C, Ad45C and Ad49C were small with entire edge, clear with no secondary growth. Those of Ad16H, Ad37H, Ad39H, Ad40H, Ad45H and Ad49H were small with diffuse, irregular edge and complete veil of secondary growth.

Patch characters. With phage dilutions producing confluent lysis three distinct types of patch were observed. The Ad2 and Ad20 phages produced similar patches with considerable secondary growth surrounded by a clear zone which disappeared as the concentration decreased. The C phages produced a completely clear patch with distinct edges. The H phages produced a patch covered by a veil of secondary growth and no outer clear zone.

Heat resistance. Typical results of the heating tests are given in Table 2.

TABLE 2.

Heat resistance tests.

Phage preparation	No. of plaques unheated	No. of plaques after heating for 30 minutes at 55° C.	60° C.	65° C.	70° C.
Ad2	181	155	81	12	—
Ad16C	75	25	—	0	0
Ad16H	425	107	2	0	0
Ad20	207	61	7	—	—
Ad37C	180	20	—	—	—
Ad37H	340	90	—	—	—
Ad39C	158	20	—	0	0
Ad39H	93	9	—	0	0
Ad40C	117	22	—	0	0
Ad40H	317	110	1	0	0
Ad45C	80	25	—	0	0
Ad45H	542	162	—	0	0
Ad49C	80	25	—	0	0
Ad49H	117	22	—	0	0

— = no plaques detected. 0 = not tested.

The plaque counts of all the phages except Ad2 were reduced more than 50 p.c. by heating for 30 minutes at 55° C. and generally no plaques appeared after heating for 30 minutes at 60° C. Ad2 was more resistant to heat and the plaque count was only slightly reduced after 30 minutes at 55°C. Greater

reductions occurred at 60° C. and 65° C. and after 30 minutes at 70° C. no plaques were detected. Similar resistance was shown by Ad2 mutant. All these phages were therefore relatively heat-sensitive.

Serological tests. Antisera were prepared against the phages Ad2, Ad16C, Ad16H, Ad20, Ad37C and Ad37H. Cross-neutralization tests were performed with all these phages and their antisera and the results are given in Table 3.

TABLE 3.

Cross-neutralization tests with phage antiserum.

Phage No.	Phage antiserum					
	Ad2	Ad20	Ad16C	Ad16H	Ad37C	Ad37H
Ad2	+	+	—	+	±	tr
Ad20	+	+	—	±	±	tr
Ad16C	—	—	+	+	+	+
Ad16H	—	—	+	+	+	+
Ad37C	—	—	+	+	+	+
Ad37H	tr	tr	+	+	+	+

The antisera were used at dilutions corresponding to their end titres as follows: Ad2 1/100, Ad20 1/100, Ad16C 1/5, Ad16H 1/10, Ad37C 1/1000, Ad37H 1/10.

+ = complete neutralization, no plaques formed.
± = at least 50 p.c. reduction in plaque count.
tr = less than 50 p.c. reduction in plaque count.
— = no neutralization, plaque count unchanged.

The C and H phages showed complete cross-neutralization. The Ad2 and Ad20 phages also showed complete cross-neutralization. The phages therefore formed two antigenic groups, one consisting of the C and H phages and the other of Ad2 and Ad20. Though distinct, the two groups were related as shown by the cross-reactivity of Ad16H, Ad37C and Ad37H antisera with Ad2 and Ad20. This cross-reaction was weaker than the reaction with the other C and H phages and was not shown by Ad16C antiserum which was not strong. Little cross-reaction was shown however between the C and H phages and Ad2 or Ad20 antiserum unless the serum concentration was increased about 100-fold when partial neutralization of both C and H phages occurred.

Production of resistant strains and lysogenicity. Strain 14 was treated with phage Ad2, Ad16C, Ad16H, Ad20, Ad37C, Ad37H, Ad49C or Ad49H. Resistant colonies were obtained readily with the H phages, less readily with Ad2 and Ad20 phages and only with difficulty with the C phages. The resistant strains produced by the H phages resembled the natural lysogenic strains carrying these phages and were lysogenic for strain 14, resistant to H and C phages and lysed by Ad2 and Ad20. The resistant strains produced by Ad2, Ad20 or the C phages varied in their susceptibility to phage action, some of them giving a lysis pattern similar to the natural lysogenic strains, others giving lysis

patterns not yet encountered in any naturally occurring strain. Lysogenicity was readily demonstrated for most of these strains. Further work on the resistance types produced by Ad2, Ad20 and the C phages is in progress and will be reported separately.

Reactions of the phages with other Salmonellas. The results of tests with the phages Ad2, Ad20, Ad37C and Ad37H on other Salmonellas are given in Table 4.

TABLE 4.

Reactions of S. adelaide *phages with other Salmonella types.*

Salmonella type tested	No. of strains	Phage used in test			
		Ad2	Ad20	Ad37C	Ad37H
S. paratyphi A	1	+	+	+	+
S. paratyphi B	2	—	—	+	+
S. typhi-murium	1	—	—	+	+
	1	—	—	—	—
S. derby	3	—	—	+	—
S. muenchen	2	—	—	+	+
S. bovis-morbificans	3	—	+	+	+
	2	—	—	+	+
S. blegdam	2	—	—	+	+
S. enteritidis	1	—	+	+	+
	1	—	—	+	+
S. anatum	1	+	+	+	+
	1	—	—	+	—
S. worthington	1	—	—	+	—
S. aberdeen	1	—	—	+	+
S. carrau	1	+	—	+	+
S. onderstepoort	1	—	—	—	—
S. waycross	3	—	—	+	—
S. adelaide, strain 14	1	+	+	+	+

Phage preparations used undiluted. + = confluent or semiconfluent lysis. — = no lysis or some plaques only.

The C and H phages acted upon a wide range of Salmonella types, but phages Ad2 and Ad20, apart from the production of a few plaques on several types, were much more restricted, especially Ad2, which acted only on one strain each of *S. paratyphi* A, *S. anatum* and *S. carrau*. Ad20 acted on *S. paratyphi* A and some strains of *S. enteritidis*, *S. bovis-morbificans* and *S. anatum*. Ad37C and Ad37H showed parallel good activity against *S. paratyphi* A, *S. paratyphi* B, *S. typhi-murium*, *S. muenchen*, *S. bovis-morbificans*, *S. blegdam*, *S. enteritidis*, *S. anatum* and *S. carrau*. The C phage had the widest range of action, lysing also strains of *S. derby*, *S. worthington*, *S. waycross* and *S. anatum* resistant to H phage. Only one strain each of *S. typhi-murium* and *S. onderstepoort* failed to lyse with the C phage. The Salmonella strains selected for testing included as far as possible some non-lysogenic strains and some representatives of any known phage groups.

Reactions of phages with other members of the Enterobacteriaceae. The results of tests with phages Ad2, Ad20, Ad37C and Ad37H on a representative range of strains from groups of the *Enterobacteriaceae* other than Salmonellas are given in Table 5.

TABLE 5.

Reactions of S. adelaide *phages with groups of the* Enterobacteriaceae *other than Salmonella.*

Organism tested	No. of strains tested	Phage used in test Ad2	Ad20	Ad37C	Ad37H
E. coli O III	3	—	—	—	—
E. coli O 55	1	—	—	—	—
Ballerup	1	—	—	—	—
Bethesda	2	—	—	—	—
Arizona	1	—	—	+	+
Sh. flexneri	1	—	—	+	+
Sh. boydii	1	—	—	—	—
Sh. dispar	1	—	—	—	—
S. adelaide, strain 14	1	+	+	+	+

Phage preparations used undiluted. + = confluent lysis. — = no lysis.

The results in Table 5 show again the greater range of activity of the C and H phages, which lysed *Sh. flexneri* and an Arizona strain, as compared with phages Ad2 and Ad20 which failed to lyse any of the organisms in Table 5. On the other hand the failure of all these *S. adelaide* phages to lyse any of the three strains of *E. coli* O III was unexpected, as Kauffmann (1952) has shown a very close antigenic relationship between this group and *S. adelaide*.

Brief description and grouping of the S. adelaide *phages.*

The four kinds of *S. adelaide* phage so far found in lysogenic strains fell into two main groups, each divisible further into two sub-groups. The two main groups have therefore been designated type 1 and type 2 and further sub-divided into types 1a, 1b, 2a and 2b. The following brief description of each suggested type is given for convenient reference.

Type 1, represented by phages Ad2 and Ad20, had distinctive plaques and patches, the plaques having an irregular edge and secondary growth more concentrated in the centre, the patches showing secondary growth and an outer clear zone; it was antigenically distinct from, though related to, type 2, produced a mutant which lysed the parent strain from which the phage was obtained, lysed strains of *S. adelaide* carrying type 2 phage and was found in naturally occurring lysogenic strains of *S. adelaide* group 1 (see Table 6). Type 1 phages were further divided into two sub-types 1a and 1b.

Type 1a (phage Ad2) was more heat resistant than type 1b, lysed strains of *S. adelaide* carrying type 1b and was found in naturally occurring lysogenic strains of *S. adelaide* group 1a (see Table 6).

Type 1b (phage Ad20) was less heat resistant than type 1a, lysed strains of *S. adelaide* carrying type 1a phage and was found in naturally occurring lysogenic strains of *S. adelaide* group 1b (see Table 6).

The mutant derived from either type 1a or type 1b lysing the parent strain was not found so far in naturally occurring lysogenic strains. In this form types 1a and 1b could be distinguished by the greater heat resistance still retained by type 1a.

Type 2, represented by all the C and H phages, lysed strains of *S. adelaide* carrying type 1 phage and was antigenically distinct from, though related to, type 1. Type 2 phages were further divided into two sub-types 2a and 2b.

Type 2a (phage Ad37H) had distinctive plaques and patches showing a complete veil of secondary growth, regularly produced a mutant, type 2b, with clear plaques and patches, readily produced resistant, lysogenic cultures from the indicator strain 14, and was found in naturally lysogenic strains of *S. adelaide* group 2 (see Table 6).

Type 2b (phage Ad37C) was a mutant of type 2a which regularly appeared after serial transfer of type 2a on the indicator strain 14, had distinctive plaques and patches which were clear, only with difficulty produced resistant, lysogenic cultures from the indicator strain 14, and was not found so far in any naturally lysogenic strain of *S. adelaide.*

Phage grouping of strains of S. adelaide. A scheme of grouping for strains of *S. adelaide* on phage lysis is given in Table 6.

TABLE 6.

Scheme of grouping for strains of S. adelaide *on phage carried and lysis pattern.*

	Phage type used in testing			
S. adelaide Group No.	type 1a (Ad2)	type 1b (Ad20)	type 2b (Ad37C)	Phage type carried
1a (strain 2)	—	+	+	1a
1b (strain 20)	+	—	+	1b
2 (strain 37)	+	+	—	2a
3 (strain 14)	+	+	+	none

In this grouping scheme type 2b phage was used in preference to type 2a as it produced the same reactions but gave much clearer lysis and patches which were easy to read. Types 1a and 1b phage were both necessary to distinguish the sub-groups of group 1. Group 1 was characterized by susceptibility to type 2 phage, group 2 by susceptibility to type 1 phage and group 3 by susceptibility to both type 1 and type 2 phages. Group 1 was subdivided into 1a and 1b on sensitivity to types 1a and 1b phage. The type of phage carried by the various

groups is also included in Table 6 and is obviously related to the lysis pattern.

In our collection of 48 strains, one strain each of groups 1a and 1b and 10 strains of group 2 occurred. The remaining strains, except one, belonged to group 3. The one exception (strain 32 already recorded by Atkinson *et al.*, 1952) showed unusual reactions and could not be grouped.

DISCUSSION.

By testing on the sensitive *S. adelaide* strain 14, the twelve lysogenic strains of *S. adelaide* each produced a single phage type and thus were considered to carry only one type of phage. Other phages might also be present but undetected by strain 14. Further tests of all *S. adelaide* strains will be made using the sensitive indicator strains of other Salmonellas under phage investigation and other phages might then be found. For the present, however, the phages of the twelve lysogenic strains were the only *S. adelaide* phages available and their division into types 1 and 2 seemed well justified, not only on their plaque and serological characters but also on their host range among other Salmonellas. The type 1 phages were more restricted than type 2 phages in their action on other Salmonellas and produced confluent lysis with undiluted preparations only on *S. paratyphi* A and certain strains of *S. carrau, S. anatum, S. enteritidis* and *S. bovis-morbificans* (including the sensitive indicator strain 18 used for phages of *S. bovis-morbificans*). They also failed to lyse any other members of the *Enterobacteriaceae* tested here. However, on *S. adelaide* strains, type 1 phages produced confluent lysis on all tested, including the two strains carrying type 1 phages (one lysed by type 1a, the other by type 1b) and the one ungrouped strain (which was lysed by type 1b). Type 1 phages were thus *S. adelaide* phages in the sense that, between them, they lysed all our *S. adelaide* strains but few other Salmonellas. Type 2 phages were not, in this sense, *S. adelaide* phages as they showed a wide range of action on other Salmonella types while failing to lyse strains of *S. adelaide* carrying type 2 phage (10 strains out of 48). Type 2b phage was almost a universal Salmonella phage as it lysed all strains of all types tested except one strain each of *S. onderstepoort* and *S. typhi-murium*. Type 2a had a somewhat more restricted range. This lysis of most other Salmonella types indicated, however, that these Salmonellas were unlikely to be carrying these type 2 phages or even closely related phages. In this sense, therefore, if they prove to be obtained almost exclusively from *S. adelaide* strains, the type 2 phages might also be regarded as *S. adelaide* phages. Type 1 and type 2 phages were not mutually exclusive nor were types 1a and 1b.

From heat resistance, and plaque size and appearance the *S. adelaide* phages probably belonged to the sub-group B of Boyd (1950) based on the original A and B types of Burnet (1932). Boyd applied these groupings to the symbiotic phages which he obtained from *S. typhi-murium*. For two of his sub-group B

phages he described "autogenous" lytic action on the parent strain when concentrated phage was propagated on the indicator strain. This "autogenous" action is similar to that which we observed with the *S. adelaide* type 1 phages. Our "autolytic" mutant appeared in undiluted preparations after serial propagation and was not present in large numbers. It could be greatly increased by propagation on the parent strain and reached titres as high as those achieved on the indicator strain. It was thus unaffected by the presence of its precursor the symbiotic phage in the parent strain. The occurrence of further different host-range mutants in the type 1 phages seemed most likely judging by plaques formed on other Salmonellas. Isolates from these plaques are at present being investigated. On the other hand the highly lytic mutant of *S. adelaide* type 2 phage (type 2b) giving completely clear (C) plaques and no secondary growth on the indicator strain, was excluded from lysis of the original parent strain by carriage of the original type 2(H) phage (type 2a).

The failure to find natural lysogenic strains containing type 2b (C) phage was probably due to the lytic nature of this variant which even when it was present in high concentration in a susceptible culture might fail to produce resistant, lysogenic colonies. In natural conditions very little of this phage would be likely to occur if its sole origin was by mutation of type 2a (H) phage. Susceptible organisms in the presence of type 2a phage were readily transformed into resistant, lysogenic forms bearing this phage which rendered them also resistant to type 2b phage. Thus most resistant strains would already bear type 2a phage and thus resist type 2b phage, and susceptible strains would be lysed by contact with type 2b phage. The two mechanisms would therefore work against the natural occurrence of resistant, lysogenic strains carrying type 2b phage.

The grouping of the phages into types and their characterization seemed desirable for future reference. Numerous Salmonella symbiotic phages are under test in this laboratory and we hope eventually to achieve some systematic grouping for the whole collection. The *S. adelaide* phages are the first of our phages to be characterized.

The grouping of the *S. adelaide* strains on lysis or phage carried was purely incidental to this study of the lysogenic strains. The grouping may, however, have practical application and epidemiological significance and the method is being applied to a larger group of strains.

SUMMARY.

From 12 lysogenic strains of *S. adelaide* four kinds of phage were isolated. These phages were divided into two types, 1 and 2, each of which was further divided into two sub-types a and b. Types 1 and 2 differed in plaque and patch morphology, in host range and in serological reactions. Serologically, however, they were related.

Types 1a and 1b each occurred in a single lysogenic strain and differed from each other in heat resistance and host range. Each phage attacked the lysogenic strain carrying the other phage.

Type 2a, which was a hazy (H) plaque phage, was the commonest and occurred in ten lysogenic strains. Type 2b was a clear (C) plaque phage which regularly appeared in type 1a preparations but was not found in any lysogenic strain.

Brief descriptions of each phage type are given. A scheme is also given for the grouping of strains of *S. adelaide* on lysis pattern using phage types 1a, 1b and 2b. Four groups emerged in this scheme and were related to the type of phage carried.

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UROPORPHYRINS

I. UROPORPHYRIN CONTENT OF NORMAL URINE

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Nicholas and Rimington (1949) showed by paper chromatography that the porphyrins of normal urines include small amounts of uroporphyrin, and even as early as 1924 Fischer and Zerweck found traces of an ether-insoluble porphyrin in 100 litres of normal urine. However, it is still often tacitly assumed that uroporphyrin does not occur in urine except in the porphyrias.

In this laboratory a number of samples of urine have been received for the diagnosis of porphyria. The recognition of acute porphyria during an attack (and sometimes during remission) from the high porphobilinogen and the high uroporphyrin content of the urine presents no difficulty, but a careful examination of the urinary samples received (e.g. from relatives of acute porphyria patients) showed detectable quantities of uroporphyrin in all cases. It was decided to measure the uroporphyrin content of normal urine, in order better to judge borderline cases.

Calcium phosphate precipitation of uroporphyrin in the presence of alkali and excess calcium ions (Sveinsson, Rimington and Barnes, 1949) brings down with the uroporphyrin from normal urine considerable amounts of other colouring matter. The amount of this present is too great in comparison with the small amount of uroporphyrin present for the Soret band of the porphyrin to be measured as described by Rimington. The colouring matter may be largely removed by esterification of the uroporphyrin followed by extraction from ether with hydrochloric acid. The Soret band can then be satisfactorily measured.

Methods.

To 10 ml. of urine, 1 ml. of glacial acetic acid is added and the coproporphyrin is removed by extracting with 20 ml. of ether. The ether is washed twice with a total of 5 ml. water and the washings added to the main aqueous layer. To this is added 0·5 ml. saturated $CaCl_2$ solution and then 40 p.c. NaOH, 1·5 ml. being added after the first permanent precipitate. The calcium salts are then spun down, washed once with methanol to remove water and dissolved in methanolic HCl. The solution is allowed to stand for at least an hour and is then poured into saturated sodium acetate overlayered with ether. The ether is washed with

[1] Working under a National Health and Medical Research Council grant.

water, Na_2CO_3 and water and extracted three times with a total of approximately 4 ml. 3N HCl. The optical densities are then read in a 1 cm. cuvette at 425 mμ, 407 mμ, and 390 mμ. The three values chosen are closer together than those used by Rimington (1949) so that the assumption of a straight absorption curve for the accompanying colouring matter is better justified and the measurements can conveniently be carried out using the glass prism of the Hilger Uvispek Spectrophotometer. To calculate the results the value of 4·9 is used for the expression

$$\epsilon^{407} - \frac{17}{35} \times \epsilon^{425} - \frac{18}{35} \times \epsilon^{390}$$

for 1 cm. layer of solution containing 1 mg. of free uroporphyrin per 100 ml. This corresponds to the distance OX in the curve A (uroporphyrin) in Fig. 1 and is proportional to the uroporphyrin concentration. This value was calculated by measuring a solution prepared by diluting one hundredfold with 3N HCl a solution of uroporphyrin octamethyl ester in neutral dioxane. The strength of the dioxane solution was checked from the value found by Stern and Wenderlein (1935) for ϵ^{499}_{M} of 1,567. This agreed well with the value of 1,556, 1,564 and 1,563 found for the preparation of uroporphyrin ester used. The strength of acid chosen, 3N, was to ensure complete extraction of the ester from ether with small quantities of acid. The height of the Soret band is somewhat less in 3N HCl than in 0·5 N HCl but the loss in sensitivity is negligible. Using 0·5N HCl a value of 6,000 was found for $\epsilon^{405}_{1\%}$ and of 1·89 was found for

$$\frac{2\epsilon^{405} - (\epsilon^{430} + \epsilon^{380})}{\epsilon^{405}}$$

(Sveinsson *et al.* quote values of 5,000 and 1·84).

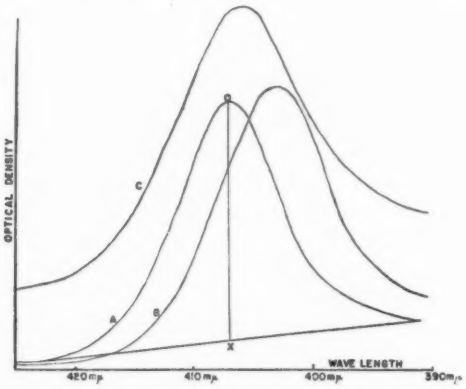

Fig. 1. Porphyrin absorption spectra. A, recrystallized uroporphyrin from a case of acute porphyria. B, coproporphyrin III. C, ether insoluble porphyrin from normal urine. All curves measured in 3N HCl. Optical density in arbitrary units.

TABLE 1.

Recovery of uroporphyrin added to normal urine.

Urine	Added μg./ml.	Found μg./ml.	Recovery p.c.
B.J.B.	0	0·018	—
	0	0·013	—
	0·017	0·027	65
	0·034	0·042	76
	0·068	0·076	88
J.F.	0	0·019	—
	0	0·015	—
	0·020	0·034	85
	0·039	0·052	90
	0·078	0·081	93
		Mean Recovery	82 p.c.

Table 1 shows the percentage recovery of porphyrin added to urine. The average recovery is 82 p.c. The identification of the pigment measured as uroporphyrin depends on the solubility behaviour of the free porphyrin and its methyl ester and on the shape and position of the band at 407 mμ in 3N HCl. Fig. 1 shows the curve from 425–390 mμ of the pigment isolated from 50 ml. of normal urine compared with the curves of coproporphyrin and of uroporphyrin. The slight shift of the maximum towards the blue may be due to a high proportion of partly decarboxylated uroporphyrins.

RESULTS.

Table 2 shows that while the daily excretion of uroporphyrin is less than half that of coproporphyrin, it is, nevertheless, appreciable, with a range of 15 to 30 μg. without allowing for a probable loss of about 20 p.c. in the estimation.

TABLE 2.

Subject	Daily coproporphyrin excretion, μg./day	Daily uroporphyrin excretion, μg./day
W.H.L.	95, 96	40*, 30*, 32
N.L.	100, 108	17, 22, 17†
W.M.P.	50	23†
W.M.P. (repeated)	—	16†, 15†
F.J.M.	—	27, 33
S.McG.	77, 69	28, 28
J.G.P.	66	11†

* 2 ml. of urine taken for analysis. † 100 ml. of urine taken for analysis.

It is now quite clear that urine contains substantial amounts of a precursor of coproporphyrin (Watson, 1951a; Eriksen, 1951). The high values for coproporphyrin obtained by Watson (1951b) for normal urines compared with those previously obtained are undoubtedly mainly due to inclusion of this precursor in the estimation and to the efficient extraction of the coproporphyrin by ethyl acetate compared with ether. It is suggested, however, that the value found may

be falsely increased by the inclusion of uroporphyrin extracted by the ethyl acetate and not completely washed out in the subsequent washings.

An attempt is now being made to isolate sufficient of the uroporphyrin from one or two normal subjects in order to crystallize it and to provide unequivocal identification and to get some concept of the isomer type.

SUMMARY.

The daily uroporphyrin excretion has been estimated in six normal subjects and shown to lie between 15 and 30 μg.

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UROPORPHYRINS

II. PRECURSORS OF UROPORPHYRIN IN ACUTE PORPHYRIA URINE

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From their early work on the urine of porphyria patients Waldenström and Vahlquist (1939) concluded that porphobilinogen can form either uroporphyrin or porphobilin, the formation of uroporphyrin being favoured by heating at pH 4. Recently Hawkinson and Watson (1952) have shown that they could separate from urine of acute porphyria a compound which gave a colour reaction with p-dimethylaminobenzaldehyde and another compound which proved to be a precursor of uroporphyrin. From their compound which reacted with the aldehyde the authors were unable to obtain uroporphyrin. The authors concluded that the precursor of uroporphyrin found in urine is not porphobilinogen. However, Westall (1952) has succeeded in isolating crystalline porphobilinogen and was able to show that it can be partly transformed to uroporphyrin.

The results to be described in the present paper suggest that the non-aldehyde-reacting precursor is itself derived from porphobilinogen.

Methods.

Uroporphyrin was estimated by measuring the height of the band at 407 mμ of the acid spectrum in 3 N HCl. As described in the preceding paper (Lockwood, 1953), measurements were taken at 425 mμ, 407 mμ and 390 mμ, and not at 430 mμ, 405 mμ and 380mμ as advocated by Sveinsson, Rimington and Barnes (1949).

Where the ratio of uroporphyrin to coproporphyrin was greater than 10, the error introduced by failing to remove coproporphyrin was less than other experimental errors. The uroporphyrin was measured either directly in the urine after acidification to make it 3 N with respect to HCl, in the 3 N HCl solutions of the calcium phosphate precipitates (Sveinsson *et al.*, 1949) or after phosphate precipitation and esterification.

RESULTS.

The first urines studied were examined solely for the purpose of diagnosing acute porphyria. The usual increase in the uroporphyrin content was found when the urine was pre-treated by acidification with HCl and heating. It was

[1] Working under a National Health and Medical Research Council grant.

observed with one patient that during the acute phase of his attack the urine contained a large quantity of porphobilinogen and that the increase in uroporphyrin on acidification and heating the urine was also considerable. As the patient recovered from the attack the uroporphyrin excretion remained comparatively high, the porphobilinogen content of the urine fell to some extent but the increase on heating the acidified urine disappeared, nor was there any increase at this stage on leaving the acidified urine standing for 24 hours in air as described by Rimington (1943).

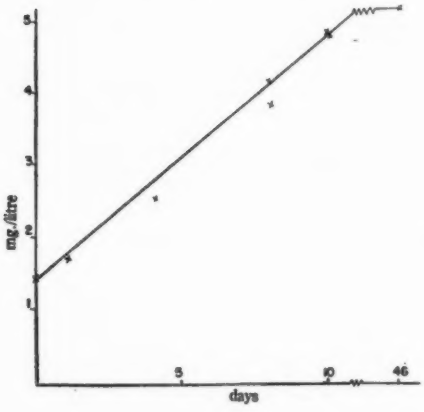


Fig. 1.

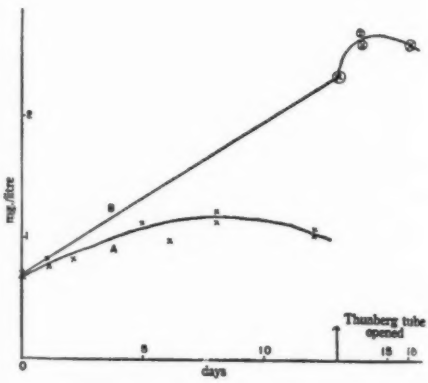


Fig. 2.

Fig. 1. The increase in the uroporphyrin content of a sample of urine which was allowed to stand in the dark at room temperature.

Fig. 2. Curve A shows the change in uroporphyrin content of a sample of urine which was allowed to stand in the dark at room temperature. Curve B gives the uroporphyrin content of a parallel sample of the same urine kept for 13 days under nitrogen and then opened and allowed to stand a further three days in the dark.

One sample of urine from the same patient had been accidentally left out of the refrigerator for a week. It was noticed that the porphyrin bands appeared to have increased in intensity. This was confirmed by estimation of the porphyrin content. Fig. 1 shows the increase of uroporphyrin on standing at room temperature in the dark. The pH was about 6.

Bacterially contaminated specimens seemed to show a more pronounced effect than those with preservative (toluol or ether). It was thought that the difference might arise from low oxygen tension produced by bacteria. This was confirmed by allowing urine to stand in a Thunberg tube; the gas space was filled with nitrogen, and alkaline pyrogallol solution put in the stopper; chloroform or toluol was added to the urine to prevent bacterial growth; another

sample of the same urine, allowed to stand in air at the same temperature, was used as a control. The increase of uroporphyrin in the sample standing under nitrogen was much greater than in that exposed to air. These results are illustrated in Fig. 2.

Another sample (from a patient in remission who had had an attack of acute porphyria over a year previously) which had a much higher content both of uroporphyrin and porphobilinogen, gave essentially similar results. The urine, because of its high pigment content, had the advantage that the uroporphyrin could be estimated by dilution of the urine with 3 N HCl, without precipitation and without ether extraction. The urine was divided into two parts and allowed to stand, with chloroform added, in the dark at room temperature, one in contact with air and the other under nitrogen. All uroporphyrin estimations were carried out by diluting with 3 N HCl (either ten-fold or one hundred-fold) and then measuring the Soret band twice, once immediately after diluting with the acid and then again after the acid-diluted urine had been allowed to stand in contact with the air in the dark for 24 hours. The uroporphyrin content at zero time (at the time the urine was divided into two parts) was 8·1 mg./litre for the immediate reading and 8·3 mg./litre for the 24-hour reading. After 11 days the immediate value for the urine standing in air was 23·7 mg./litre and the 24-hour value was 25·1 mg./litre. On the 12th day the

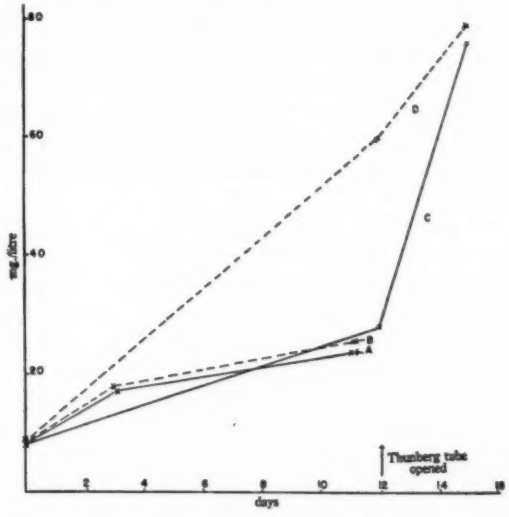


Fig. 3. The effect of incubation under nitrogen and of exposure to air. All estimations were carried out by diluting the urine 100-fold with 3 N HCl without any other treatment.

Curve A: Urine standing in the dark at room temperature and the Soret band measured immediately after diluting with acid.

Curve B: The same as curve A but the readings taken at the end of 24 hours' standing in air after diluting.

Curve C: Urine standing in the dark under nitrogen and the Soret band read immediately after diluting with acid.

Curve D: The same as curve C but the readings taken at the end of 24 hours' standing in air after diluting.

urine standing under nitrogen was exposed to air and a sample immediately diluted with acid for estimation. The immediate reading gave a value of 27·8 mg./litre and the 24-hour reading a value of 59·2 mg./litre. After air had been admitted to this sample of urine it was allowed to stand in air and the uroporphyrin content estimated again after three days, that is 15 days after it had first been put under nitrogen. The immediate reading then gave a value of 75·1 mg./litre and the 24-hour reading a value of 78·2 mg./litre. These results are illustrated in Fig. 3. Fig. 4 shows the increase in porphyrin content between the immediate reading and the 24-hour reading where this increase was greatest, i.e. immediately after opening the urine which had been allowed to stand under nitrogen. Calcium phosphate precipitation of the uroporphyrin at intervals after allowing the undiluted and unacidified urine to stand, showed a similar increase in the precipitable uroporphyrin, parallel but not quite identical with that shown by the acidified and diluted urine without calcium phosphate precipitation.

These results were confirmed in general for all urines containing porphobilinogen although quantitatively there were considerable differences between the amounts of uroporphyrin formed under nitrogen and the amounts formed after the admission of air, and an increase after admitting air could only be shown convincingly with urines with a high porphobilinogen content. Fig. 2, however, while not very significant, does suggest some rapid uroporphyrin formation after admission of air.

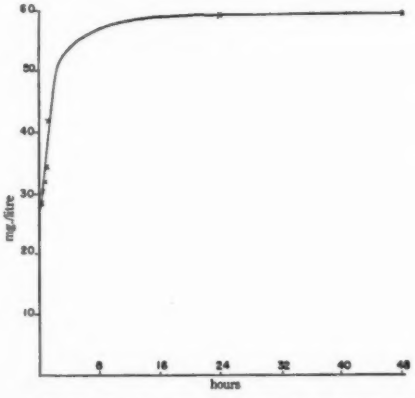


Fig. 4. The increase in height of the Soret band, expressed as mg./litre of uroporphyrin, after urine which had been allowed to stand in the dark under nitrogen for 12 days was diluted 100-fold with 3 N HCl and allowed to stand in air (cf. Fig. 3).

Gibson and others (1950) claimed that preformed uroporphyrin differs somewhat from that found by boiling the urine at pH 3·7. From the quoted differences of the melting points of the coproporphyrins obtained by decarboxylation the difference of the uroporphyrins was due probably to a higher proportion of type III in that formed by boiling. A 9 ml. sample of urine was therefore allowed to stand for 3 weeks under nitrogen and then several days in air to give maximum conversion of the uroporphyrin content. Originally the total uroporphyrin content of the sample was 38 μg. The final content was

680 μg. This was precipitated with calcium phosphate, chromatographed first on heavy magnesium carbonate and then on magnesium oxide using benzene methanol, 50 : 1, and recrystallized 6 times from methanol by dissolving in chloroform and repeatedly boiling to a small volume and adding methanol till the chloroform had been completely boiled off. The losses at each stage were calculated and the recovery after the final recrystallization was 390 μg. Since the urine originally contained only 38 μg. of uroporphyrin, at least 90 p.c. of the final recrystallized uroporphyrin must have been subsequently formed uroporphyrin even if the subsequently formed uroporphyrin was preferentially lost in the purification. The appearance of the crystals and the melting point (255–260° C.) was the same as that of the uroporphyrin isolated from the fresh urine. An attempt was made to decarboxylate the uroporphyrin formed but the amount was too small to separate the crystalline isomers and all that was possible was to identify the porphyrin formed, by solubility in ether acetic acid and by spectrum, as coproporphyrin.

DISCUSSION.

The evidence brought forward in this paper shows that there are three reactions occurring in the urine of acute porphyrics leading to the formation of uroporphyrin:

a. There is the slow formation of uroporphyrin in urine under normal or low oxygen tension but the amount of uroporphyrin formed is greater under low oxygen tension;

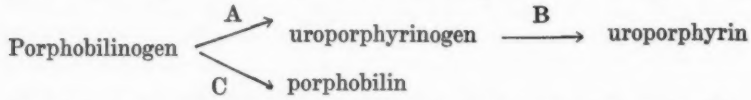
b. There is a rapid formation of uroporphyrin from a precursor under normal oxygen tension which is accelerated by acidification;

c. There is a slow formation under low oxygen tension of the precursor in reaction *b*.

It is clear that the reaction *b*, the rapid transformation of a uroporphyrinogen into uroporphyrin, is an oxidation by molecular oxygen and it is probable that the formation of this precursor (reaction *c*) is a non-oxidative process and that reaction *a* is a combination of oxidative reaction *b* and of the non-oxidative reaction *c*. If it is assumed that the conversion of the uroporphyrinogen to uroporphyrin requires 1·5 molecules of O_2 per molecule of uroporphyrin formed, then there would be 25 μg. of porphyrin formed per μl. of oxygen. Several microlitres of oxygen could well have remained in solution and in the gas space in the Thunberg tubes despite several flushings with nitrogen. This would explain the formation of porphyrin in reaction *a*.

Finally it is suggested as an hypothesis that fits the results described in the present paper and those described by Hawkinson and Watson (1952) and by Westall (1952) that porphobilinogen is slowly transformed by a non-oxidative process to a uroporphyrinogen which can then rapidly give rise to uroporphyrin.

In the presence of oxygen, however, porphobilinogen can, by an alternative reaction, be transformed to porphobilin, according to the following scheme.



Reaction A is considered to be favoured by elimination of oxygen either by heating the solution or by holding it under nitrogen, and reaction C to be favoured by allowing to stand in air at room temperatures. Waldenström and Vahlquist (1939), in describing the reactions of porphobilinogen, claimed that heating with alkali altered its nature so that, although still reacting with p-dimethylaminobenzaldehyde, the condensation product given has a slightly different spectrum and the altered porphobilinogen no longer gives uroporphyrin. This is confirmed by Westall, who showed that, while ammoniacal solutions of porphobilinogen give some uroporphyrin on boiling, sodium hydroxide solutions give porphobilin only. Hawkinson and Watson, in preparing porphobilinogen free from uroporphyrin and the non-aldehyde-reacting precursor, treated their solutions with calcium chloride and sodium hydroxide to precipitate the uroporphyrin with the phosphates. Their failure to obtain uroporphyrin from this porphobilinogen could be due to alteration by the sodium hydroxide, even in the cold.

Uroporphyrin from the urine of acute porphyrics, "Waldenström uroporphyrin," has consistently been shown to contain porphyrins both of series I and series III, and these two only. The isolated crystalline uroporphyrin formed from the precursors in urine was insufficient to identify its type by decarboxylation and identification of type of the coproporphyrin formed. However, the isolated porphyrin had the crystalline appearance and melting point (258° C.) of typical "Waldenström uroporphyrin." It is reasonable therefore to assume that the uroporphyrin formed from the precursors contains both uroporphyrin I and urporphyrin III. Since no single dipyrrolic precursor could give rise to uroporphyrin III and since any mixture of dipyrrolic pigments, which could give rise to uroporphyrin III and uroporphyrin I, would also give rise to uroporphyrin II, the evidence that porphobilinogen is a tetrapyrrolic compound becomes very strong.

However, a suggestion must be considered that porphobilinogen is a tripyrrolic chain with all three pyrrole rings orientated in the same sense, one molecule of which is capable of capturing from a second molecule one pyrrole ring to form a tetrapyrrolic chain. According to the orientation of the captured pyrrole ring, the tetrapyrrolic chain could then close to give porphyrins of type I or type III. While this suggestion is rather far-fetched, it would be able to explain a mixture of isomers I and III arising from porphobilinogen without the need for postulating the porphobilinogen as a mixture of isomers.

SUMMARY.

Experiments are described which demonstrate that not only can uroporphyrin be formed in the urine of acute porphyrics after it has been passed, but that this occurs in stages, probably porphobilinogen giving rise to uroporphyrinogen.

The formation of uroporphyrinogen from porphobilinogen is shown to occur in the absence of oxygen and uroporphyrinogen can then be converted through oxidation by molecular oxygen to uroporphyrin.

These findings are discussed reconciling recent apparently contradictory findings concerning porphobilinogen as a precursor of uroporphyrin.

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THE OCCURRENCE OF SALMONELLA TYPES IN AUSTRALIA[1]. 10.

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(Accepted for publication 21st April, 1953.)

The present paper describes 874 Salmonellas received from all over Australia. Nearly 500 strains were *S. typhi-murium*. The remainder included 35 different types, of which *S. bovis-morbificans* (70 strains) and *S. derby* (40 strains) were the commonest. In earlier papers of this series (Atkinson *et al.*, 1952) we also noted that these two types occurred relatively frequently. However, in the present group larger numbers than previously found were recorded for *S. anatum* (37 strains), *S. chester* (29 strains), *S. newport* (23 strains) and *S. meleagridis* (20 strains).

The types found and their number and origin are recorded in Table 1.

TABLE 1.

Salmonellas described in this paper.

Salmonella	Total No. of strains	Where isolated	Source	No. of strains	Type of illness
S. stanley	1	Queensland	rabbit spleen	1	from laboratory stock
S. rubislaw	1	Queensland	water-dragon	1	died after some months in captivity
S. new-brunswick	1	Queensland	child	1	gastro-enteritis
S. oslo	1	Queensland	chicken	1	25 p.c. losses in flock
S. birkenhead	1	Queensland	jew lizard	1	died with enteritis
S. glostrup	1	Queensland	child	1	gastro-enteritis
S. senftenberg	1	South Australia	adult	1	gastro-enteritis
S. eastbourne	1	Queensland	child	1	gastro-enteritis
S. moscow	2	Queensland	child	2	gastro-enteritis, *S. newport*, later as carrier
S. bareilly	2	Queensland	adult	1	gastro-enteritis
			child	1	gastro-enteritis
S. saint-paul	2	Queensland	child	1	gastro-enteritis
		New South Wales	child	1	gastro-enteritis
S. berta	2	New South Wales	children	2	gastro-enteritis
S. kottbus	3	Queensland	horse peri-cardial fluid and stomach lesions	1	used for poison plant tests
			child	1	gastro-enteritis
		New South Wales	child	1	gastro-enteritis

[1] This work was assisted by a grant from the National Health and Medical Research Council, Canberra.

TABLE 1 (continued).

Salmonella	Total No. of strains	Where isolated	Source	No. of strains	Type of illness
S. paratyphi C	4	Queensland	children (2)	3	gastro-enteritis, one died
			human	1	no history
S. heidelberg	4	Queensland	child	1	gastro-enteritis
		New South Wales	children	2	gastro-enteritis
		South Australia	milk	1	country milk causing gastro-enteritis
S. lexington	4	Queensland	chicken	3	from flock suffering losses
			bandicoot	1	died in captivity
S. paratyphi B	4	New South Wales	child	1	in outbreak at migrant camp, 5 died
		South Australia	children	2	gastro-enteritis
		Victoria	child	1	gastro-enteritis
S. orion	5	Queensland	child	1	gastro-enteritis
		New South Wales	child	1	gastro-enteritis
			adult	1	no history
		South Australia	child	1	gastro-enteritis
		New Zealand	human	1	no history
S. javiana	5	Queensland	children	5	gastro-enteritis, enteric fever, two healthy carriers
S. newington	5	Queensland	pig	1	from herd suffering losses
		New South Wales	egg pulp	4	prepared in South Australia
S. give	7	Queensland	duck	1	from flock suffering losses, *S. typhi-murium* also
			adult	1	gastro-enteritis
			chickens	5	from three flocks suffering losses, *S. anatum* also in one
S. potsdam	8	Queensland	children	2	gastro-enteritis, *S. typhi-murium* and *Sh. sonnei* also from one, *E. coli* O III and *Sh. sonnei* from other
			lizard	1	died in captivity
			lamb	1	neo-natal fatality in lambing trial
			sheep	2	experimental sheep being depleted of vitamin A
			cow	1	marked gastro-enteritis
		New South Wales	child	1	gastro-enteritis
S. london	8	Queensland	chickens	7	from three flocks suffering losses
			adult	1	healthy carrier
S. cholerae-suis	8	Queensland	pigs	8	from six different farms suffering losses, one salmonellosis
S. worthington	8	Queensland	chickens	8	from three flocks suffering losses, *S. meleagridis* also from one
S. oranienberg	8	Queensland	children	2	one gastro-enteritis, other healthy carrier
			chicken	1	from flock suffering losses
		New South Wales	egg pulp	5	prepared in South Australia
S. bonariensis	9	Queensland	child	1	gastro-enteritis
			chickens	4	blotchy livers only at P.M.
			lizards	3	one healthy, others died in captivity
		South Australia	adult	1	gastro-enteritis
S. muenchen	13	Queensland	adult	1	gastro-enteritis, died
			child	1	gastro-enteritis
			chickens	5	from one hatchery and two flocks suffering losses
			lizard	1	died of filariasis, no enteritis, *S. chester* also
			jew lizard	1	died in captivity
		South Australia	child	1	no history
			adult	2	gastro-enteritis
			kangaroo	1	found dead

TABLE 1 (continued).

Salmonella	Total No. of strains	Where isolated	Source	Total No. of strains	Type of illness
S. meleagridis	20	Queensland	chickens	10	from two flocks suffering losses, *S. worthington* also in one, or died in feeding trial
			bull	1	died with scours
			foal	1	culture from intestine
		New South Wales	adult	1	gastro-enteritis
			child	1	gastro-enteritis
			liquid egg	4	no history
			dog	1	no history
		South Australia	sheep	1	abortion*
S. adelaide	20	Queensland	children	10	gastro-enteritis, one with pertussis died. Sonne or Flexner dysentery or *S. typhi-murium* also from some
			lizards	3	two healthy, one died in captivity
			sewer rat	1	healthy
		New South Wales	children	4	gastro-enteritis
		Victoria	children	2	gastro-enteritis, or appendicitis
S. newport	23	Queensland	children	17	gastro-enteritis and three healthy carriers
			lamb	1	liver abscesses
			sheep	1	died with *Verbesina encelioides* poisoning
			pig	1	enteritis and pneumonia in a herd
		New South Wales	children	2	gastro-enteritis
		Victoria	child	1	gastro-enteritis
S. cholerae-suis var. *kunzendorf*	28	Queensland	pigs	24	enteritis, pneumonia, arthritis, lung abscesses, from various properties suffering losses
			cat	1	died with broncho-pneumonia
		South Australia	pigs	3	diseased, paratyphoid
S. chester	29	Queensland	children	17	gastro-enteritis, some also Flexner dysentery, *S. muenchen* or *S. bovis-morbificans*, one died, one healthy carrier
			jew lizards	3	died with enteritis
			lizards	2	one healthy, other died of filariasis with *S. muenchen* also
			dog	1	symptoms of encephalitis
		New South Wales	child	2	meningitis
			children	2	gastro-enteritis
		South Australia	children	2	gastro-enteritis
S. anatum	37	Queensland	children	8	gastro-enteritis
			sheep	1	died after trucking, *S. typhi-murium* also
			duckling	1	enteritis causing losses in flock
			bovine intestine	1	inflammation
			pig	1	died
			dog	1	diarrhoea after whelping
			chickens	17	from 9 flocks suffering losses, *S. muenchen*, *S. pullorum* or *S. give* in some also
		New South Wales	children	4	gastro-enteritis
			adult	1	relapsing entero-colitis
		South Australia	child	1	gastro-enteritis
		New Zealand	human	1	no history
S. derby	40	Queensland	children	8	gastro-enteritis and one healthy carrier
			adult	1	typhoid fever
			guinea pig	3	laboratory stock
			bovine liver	1	died of arsenic poisoning
			ducklings	3	from flock suffering losses
			sheep heart blood	1	died after trucking, *S. typhi-murium* also
			chickens	8	from 4 flocks and one hatchery suffering losses
		New South Wales	babies	5	from outbreak of neo-natal diarrhoea with 7 deaths
			children	6	gastro-enteritis
		South Australia	children	4	gastro-enteritis

TABLE 1 (continued).

Salmonella	Total No. of strains	Where isolated	Source	No. of strains	Type of illness
S. bovis-morbificans	70	Queensland	children	14	gastro-enteritis, *S. chester*, *S. typhi-murium* or Flexner dysentery also in some
			adults	4	gastro-enteritis, endocarditis
			chickens	7	from three flocks suffering losses, *S. meleagridis* also from one
			mice	2	laboratory stock
		New South Wales	children	22	gastro-enteritis
			adults	7	five healthy carriers, two gastro-enteritis, one died
			ward dust	1	from isolation ward
			dog intestine	1	died of distemper
		South Australia	children	9	gastro-enteritis
			adults	2	gastro-enteritis, pneumonia and fever
		Western Australia	sheep	1	died after travelling
S. typhi-murium	488	Queensland	children	143	mild to severe gastro-enteritis, at least seven died, Sonne or Flexner dysentery, *E. coli* O III, *S. oranienberg* or *S. potsdam* also from some
			adults	9	gastro-enteritis, typhoid
			ice-cream	1	home-made, caused gastro-enteritis
			guinea pigs	3	laboratory stock
			ducklings	24	from 18 flocks suffering losses
			sheep	10	died after trucking, *S. anatum* also from one
			pigs	7	pneumonia, haemorrhages, causing losses, septicaemia, one carrier
			horse	1	no history
			dog	1	diarrhoea
			chickens	13	from 11 flocks suffering losses
			cattle	12	milk fever, lead poisoning, scouring
			cat	1	slight congestion of intestine
			mouse	1	laboratory stock
		New South Wales	children	137	gastro-enteritis, at least five died, one meningitis, one encephalitis, two hospital outbreaks, two healthy carriers
			adults	7	gastro-enteritis and one healthy carrier
			liquid egg	1	no history
			sheep	1	diarrhoea, died
			cow	1	diarrhoea in herd
			canary	1	haemorrhagic enteritis
		South Australia	children	65	gastro-enteritis
			adults	6	gastro-enteritis or fever
			sheep	15	salmonellosis†, died after trucking or slaughtered at abattoirs
			magpies	2	in outbreak of salmonellosis†
			water	1	in outbreak of salmonellosis†
			guinea pigs	4	laboratory stock
			kangaroo	1	enteritis
		Victoria	adults	6	gastro-enteritis
			foals	10	from outbreaks on several properties
			calf	1	no history
		Western Australia	children	2	gastro-enteritis, meningitis
			foal	1	intense colitis

* This case was described by Rae and Wall (1952). † This outbreak was described by Watts and Wall (1952).

In Table 1 the rarer types, of which only one or two strains were received, are placed first, and the other types follow in order of numbers found. *S. typhi-murium*, with 488 strains, far outstripped all other types in prevalence and accounted for more than half the cultures received. In Table 2 the distribution of the types between human and other material is given.

TABLE 2.

Distribution of Salmonella types in human and animal material.

Salmonella	Number of strains from animals or other sources	No. of strains from man	Total No. of strains
S. stanley	1 (rabbit)	0	1
S. rubislaw	1 (water-dragon)	0	1
S. new-brunswick	0	1	1
S. oslo	1 (chicken)	0	1
S. birkenhead	1 (jew lizard)	0	1
S. glostrup	0	1	1
S. senftenberg	0	1	1
S. eastbourne	0	1	1
S. moscow	0	2	2
S. bareilly	0	2	2
S. saint-paul	0	2	2
S. berta	0	2	2
S. kottbus	1 (horse)	2	3
S. paratyphi C	0	4	4
S. heidelberg	1 (milk)	3	4
S. lexington	4 (chickens, bandicoot)	0	4
S. paratyphi B	0	4	4
S. orion	0	5	5
S. javiana	0	5	5
S. newington	5 (pig, egg pulp)	0	5
S. give	6 (chickens, ducks)	1	7
S. potsdam	5 (lizard, cow, sheep)	3	8
S. london	7 (chickens)	1	8
S. worthington	8 (chickens)	0	8
S. oranienberg	6 (chicken, egg pulp)	2	8
S. bonariensis	7 (chickens, lizards)	2	9
S. muenchen	8 (chickens, lizards, kangaroo)	5	13
S. meleagridis	18 (chickens, bull, foal, dog, sheep, liquid egg)	2	20
S. adelaide	4 (lizards, rat)	16	20
S. newport	3 (sheep, pig)	20	23
S. chester	6 (lizards, dog)	23	29
S. cholerae-suis (including var. *kunzendorf*)	36 (pigs, one cat)	0	36
S. anatum	22 (chickens, duck, pig, dog, sheep, bovine)	15	37
S. derby	16 (chickens, ducks, sheep, guinea pig, bovine)	24	40
S. bovis-morbificans	12 (chickens, mice, dog, sheep, dust)	58	70
S. typhi-murium	113 (chickens, ducks, guinea pigs, sheep, pigs, horses, dog, cat, cattle, mouse, magpies, kangaroo, canary, ice-cream, liquid egg, water)	375	488

The single strains of *S. stanley*, *S. rubislaw*, *S. oslo* and *S. birkenhead*, the four strains of *S. lexington*, the five strains of *S. newington*, the eight strains of *S. worthington* and the 36 strains of *S. cholerae-suis* (including *kunzendorf*) all came from animal material and were not isolated from man. On the other hand the single strains of *S. new-brunswick*, *S. glostrup*, *S. senftenberg* and

S. eastbourne, the two strains of *S. moscow*, *S. derby*, *S. saint-paul* and *S. berta*, the four strains of *S. paratyphi* B and *S. paratyphi* C and the five strains of *S. orion* and *S. javiana* all came from human material only. All the other types were obtained from both man and animals. From poultry and egg pulp *S. oslo*, *S. lexington*, *S. newington*, *S. give*, *S. london*, *S. worthington*, *S. oranienberg*, *S. bonariensis*, *S. muenchen*, *S. meleagridis*, *S. anatum*, *S. derby*, *S. bovis-morbificans* and *S. typhi-murium* were identified. From dogs *S. meleagridis*, *S. chester*, *S. anatum* and *S. bovis-morbificans* were obtained. Lizards and a water-dragon yielded *S. rubislaw*, *S. birkenhead*, *S. potsdam*, *S. bonariensis*, *S. muenchen*, *S. adelaide* and *S. chester*. Sheep and cattle provided strains mainly of *S. typhi-murium*, *S. derby*, *S. anatum* and *S. meleagridis*. Few Salmonellas came from horses and included *S. kottbus*, *S. meleagridis* and *S. typhi-murium*.

The majority of strains of all types obtained from man came from cases of gastro-enteritis mainly in children (see Table 1). A number of cases showed mixed infections involving other Salmonellas, *Sh. sonnei*, *Sh. flexneri* or *E. coli* O III and a considerable number of healthy carriers were detected. The commonest type found in human infections was *S. typhi-murium* (375 strains) and next, though far fewer in numbers, came *S. bovis-morbificans* (58 strains). We have noted this prevalence in all the previous groups recorded although the actual number of strains of *S. bovis-morbificans* was much greater in our last group (Atkinson *et al.*, 1952) on account of the inclusion of a large epidemic due to this type. The next commonest type, as we found previously, was *S. derby* (24 strains), followed by *S. chester* (23 strains), *S. newport* (20 strains), *S. adelaide* (16 strains) and *S. anatum* (15 strains).

The 36 types found here were distributed among the O groups of the Kauffmann-White scheme as follows:

Group A	none	
Group B	7 types	568 strains (including 488 *S. typhi-murium*)
Group C_1	7 types	60 strains (including 36 *S. cholerae-suis*)
Group C_2	6 types	119 strains (including 70 *S. bovis-morbificans*)
Group D	4 types	10 strains
Group E_1	6 types	81 strains (including 37 *S. anatum*)
Group E_2	2 types	6 strains
Group E_3	1 type	1 strain
Group F	1 type	1 strain
Group G	1 type	8 strains
Further groups	1 type	20 strains (all *S. adelaide*)

Thus the great majority of types identified here belonged to Groups B, C and E, to which 835 strains were allotted out of the total of 874. The remaining 39 strains included 20 *S. adelaide*, a rare type not allocated to a specific group, which we have found relatively common in Australia. In other parts of the world the majority of Salmonellas fell into the first five Groups A, B, C, D and E (Kauffmann, 1951). In Australia Groups A and D were relatively rarely represented but the other three, B, C and E, were commonly found.

SUMMARY.

Among 874 Salmonellas obtained from man and animals and typed in this laboratory 835 fell into Groups B, C and E.

S. typhi-murium far outnumbered any other type.

Other relatively common types were *S. bovis-morbificans*, *S. derby*, *S. anatum*, *S. cholerae-suis*, *S. chester*, *S. newport*, *S. adelaide* and *S. meleagridis*. In all, 35 different types were identified.

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473

PSITTACOSIS AMONG BIRDS IN SOUTH AUSTRALIA

I. A SURVEY OF INFECTION IN SOME COMMON SPECIES IN 1951 AND 1952

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(Accepted for publication 12th April, 1953.)

In 1933 Meyer and Eddie reported that psittacosis was widespread among budgerigars in aviaries in California.

They therefore imported a fresh stock of recently captured Australian budgerigars in the hope of obtaining a disease-free stock, but found some of the birds already infected on arrival in California (1934).

Burnet (1935) then investigated Australian psittacines, and found that infection was widespread.

He obtained birds from widely separated areas over most of Australia, and found infection in 50 out of 274 birds examined. Although there were infections both in wild birds and in those obtained from dealers, the infection rate in the cage birds was notably higher than that in the wild birds.

The infected birds had come from Queensland, South Australia and Victoria.

In 1936 Burnet and MacNamara reported psittacosis in man, acquired from the white cockatoo *(Kakatao galerita)* and in 1938 Tremain recorded deaths from psittacosis in seven species of parrot captured in New South Wales, and in two species of finch captured in the Northern Territory, either in transit or while in quarantine in New South Wales.

During 1938 an epizootic of psittacosis occurred in wild psittacines in southern Australia (Burnet, 1939). In May to July wild bird fatalities were observed in Victoria, particularly in King parrots *(Aprosmictus scapularis)* and Crimson rosellas *(platycerus elegans)*; in October deaths among Eastern rosellas *(P. eximius)* were observed near Launceston, Tasmania, and in October and November a highly fatal epizootic occurred in South-East of South Australia.

Mr. R. F. Bellchambers has told us that in January, 1939, he observed parrots falling dead from the trees at Humbug Scrub near Adelaide, South Australia, but he attributed these deaths to the exceptional heat wave at the time, and no investigations were made on these birds.

Since the time when these investigations were made, it has become apparent that the psittacosis group of viruses affects not only psittacines but a wide variety of other birds in the wild. The infection of such species had not been investigated in Australia.

[1] Assisted by a grant from the National Health and Medical Research Council.

Further, the occurrence of the 1938 epizootic shows that the equilibrium between the viruses and their hosts is unstable. We have, therefore, commenced surveys of the degree of infection of some of the commoner species of bird in the settled areas of South Australia in an attempt to find the amount of infection present, the characteristics of the strains of virus isolated and whether any gross variations in the degree of infection of different species can be observed.

In this paper we report on the birds investigated in autumns of 1951 and 1952.

Materials and Methods.

Birds.

Wild birds were either collected specifically for the investigation, obtained from the Veterinary division of this Institute which had collected them for another purpose, or were received from the South Australian Museum which had received them from a variety of sources.

Domestic pigeons were bought in from fanciers in the Adelaide metropolitan area, and aviary-bred birds and domestic fowls were obtained from aviculturalists in the Adelaide metropolitan area.

The birds included as wild birds had either been shot or were killed within 24–48 hours of capture. In these cases the results recorded were obtained entirely from virus isolation.

In the domestic pigeons and fowls, in most instances, the diagnosis of infection or absence of infection was made on serological grounds.

Most of the birds investigated were collected in an area bounded in the south by the Adelaide metropolitan area, extending to Burra in the north, and west as far as the coast of St. Vincent's Gulf. Small numbers of birds were received from Hawker, farther to the north, Karoonda and Bordertown to the east, and from Hindmarsh Valley and Milang in the south. (See Fig. 1.)

Fig. 1. Map of districts from which birds have been examined.

Virus isolation.

Birds, after they had been collected, were brought to cold store as soon as possible. Some of the smaller birds were placed on ice in a large vacuum flask immediately. The birds were

weighed and measured; the spleen was also measured, and a portion of the liver was removed. The tissues were ground with sand, extracted in the cold, and centrifuged to deposit debris. According to the number of birds to be dealt with at any one time, these extracts were either inoculated separately, or in pools representing two to four birds, into groups of four sixth-day embryonated hens' eggs by the yolk sac route. The eggs were observed for up to 10 days, and if virus was not isolated on the first passage, two further blind passages were carried out before the material was discarded as non-infectious. In most cases 500 units of streptomycin per ml. were added to the material before inoculation of 0·25 ml. into the eggs. In the few cases in which the material proved to be contaminated with streptomycin-resistant bacteria, the material was inoculated intracerebrally in 0·03 ml. volumes into weaned mice. In no case did we obtain a strain of virus from such material.

When strains had been established in the hen's egg, they were tested for mouse pathogenicity by intracerebral, intranasal, and intraperitoneal inoculation.

Serological tests.

The serological tests used were the direct and indirect complement fixation tests.

The antigen was prepared by the differential centrifugation of a yolk sac preparation of our standard laboratory strain of psittacosis of parrot origin. The antigen was used after having been steamed. All reagents were used in 0·2 ml. quantities. Serum was inactivated by heating to 56° C. for 30 minutes. (In the few fowl sera tested this proved satisfactory and it is almost invariably satisfactory with pigeon sera.)

In the direct test, serial dilutions of the unknown sera were made, beginning at 1:5. Two and one-half minimal haemolytic doses (MHD) of complement were added (the titre of the complement was taken as the dilution giving 50 p.c. haemolysis in the presence of the test antigen), and an optimal dose of antigen which had been previously decided by a checkerboard titration with a standard positive serum. Fixation was allowed to take place for one hour at 37° C. Three p.c. sheep's red blood corpuscles sensitized with 5 MHD of haemolysin were then added, and the tubes were returned to the water bath for a further 30 minutes. The results were read after the cells had been deposited by centrifugation in those tubes showing intermediate degrees of haemolysis. Significant fixation was regarded as present when over 50 p.c. of the cells remained unhaemolysed. No serum was regarded as positive which did not give complete fixation at a dilution of 1:5 or higher.

In the indirect test, originally developed by Rice (1948) and Karrer, Meyer and Eddie (1950), the unknown serum was diluted serially from 1:5, and two MHD of antigen were added. This mixture was allowed to incubate for 1 hour at 37° C. The two and one-half MHD of complement were added, and two complement fixing doses of a known positive serum. This mixture was incubated for 1 hour at 37° C. before the addition of sensitized R.B.C. The result was read as in the previous test after 30 minutes' incubation at 37° C. and centrifugation to deposit remaining cells. In this test bird sera incapable of fixing guinea-pig complement, but capable of combining with antigen, inhibit the subsequent fixation of complement by a known positive serum. In our tests sera capable of completely inhibiting complement fixation at a dilution of 1:5 or higher were regarded as positive.

RESULTS.

Wild Psittacines.

The results of our investigations on wild birds are shown in Table 1. The areas from which the birds were collected in each year are also shown on the Table.

TABLE 1.

Wild Birds.

Species	Areas examined	1951 No. tested	1951 No. positive	Areas examined	1952 No. tested	1952 No. positive
Galah (*Kakatao roseicapilla*)	Burra, Eudunda	20	0	Balaklava, Eudunda Hawker	23	0
Adelaide Rosella (*Platycercus adelaidae*)	Adelaide, Burra, Eudunda	24	6*	Hindmarsh Valley, Mt. Crawford, Adelaide, Eudunda, Hamley Bridge	36	5*
Red Rump Parrot (*Psephotus haematonatus*)	Burra, Eudunda	13	1	Balaklava, Eudunda, Hawker	19	3*
Mallee Ring Neck (*Barnardius barnardi*)	Burra	2	0	Hawker	2	0
Eastern Rosella (*Platycercus eximius*)	Bordertown	1	0	—	—	—
Purple Crowned Lorikeet (*Glossopsitta porphyriocephala*)	—	—	—	Adelaide	1	0
Sparrow (*Passer domesticus*)	Roseworthy	30	1†	Roseworthy	30	0
White-backed Magpie (*Gymnorhina hypoleuca*)	Burra, Hamley Bridge	12	0	Hamley Bridge	2	0
Raven (*Corvus coronoides*)	Burra	1	0	Karoonda	5	0
Crested Pigeon (*Ocyphaps lophotes*)	Hamley Bridge	8	0	Hamley Bridge	11	0
Indian Dove (*Streptopelia suratensis*)	Adelaide	8	0	Adelaide	9	0
Starling (*Sturnus vulgaris*)	—	—	—	Adelaide, Balaklava Milang	16	0
Little Falcon (*Falco longipennis*)	Burra	1	0	—	—	—
Wedge-tailed Eagle (*Uroaetus audax*)	Adelaide	1	0	—	—	—
Black shoulder Kite (*Elanus axillaris*)	—	—	—	Eudunda	1	0
Noisy Miner (*Mysantha melanocephala*)	—	—	—	Mt. Crawford	2	0
		121	8*		157	8*

* These figures include pools of 2 to 4 birds and assume that only one bird was infected in any one pool.
† This strain was isolated from a pool of 14 spleens and assumes that only one was infected.

Our investigations have only covered a large enough number of birds of three of the six psittacine species tested for them to be of any significance, and these results showed no significant differences between the two years. Both the Adelaide rosella *(Platycercus adelaidae)* and the red rump parrot *(Psephotus haematonatus)* showed a substantial proportion of infected birds.

The numbers examined are not sufficient to allow us to demonstrate any differences in the amount of infection in the various districts from which the birds were obtained.

In contrast to these parrots, the galahs *(Kakatao roseicapilla)* which have been examined so far have not been infected.

The total number of specimens (43) was quite small, but we are at least justified in saying that infection of galahs was certainly not common in this

State, and that infection, if it did occur here, was much less intense than that of the rosellas and red rump parrots.

The four Mallee ring necks *(Barnardius barnardi)* and the single specimen of Eastern rosella *(P. eximius)* and purple-crowned lorikeet *(Glossopsitta porphyriocephala)* did not yield strains of virus.

In most cases where virus was isolated, the spleen of the bird tested was found to be enlarged, but on two occasions pools of spleens from Adelaide rosellas, which had been regarded as normal, did give strains of virus. In our series of galahs, two were found with grossly enlarged spleens, but neither gave a strain of psittacosis virus.

Wild birds other than psittacines.

The results with non-psittacine birds were in sharp contrast to those given above. More than ten specimens were examined from 5 species of birds. No infected birds were found among 14 white-backed magpies *(Gymnorhina hypoleuca)*, 19 crested pigeons *(Ocyphaps lophotes)*, 17 Indian doves *(Streptopelia suratensis)* or 16 starlings *(Sturnus vulgaris)*. One strain was obtained from a pool of 14 spleens from sparrows *(Passer domesticus)*. Three of these spleens were definitely enlarged. This was the only strain isolated from 60 sparrows, including 10 with enlarged spleens. The 1952 specimens were examined in smaller pools than those used in the earlier series, from which a strain was isolated. This strain was notable in having an extremely low pathogenicity for mice intracerebrally, intranasally or intraperitoneally, and could never have been isolated in mice. Mice killed on the tenth day after intranasal inoculation of a 10 p.c. suspension of heavily infected 4th yolk sac passage material all showed some lung consolidation and histological preparations showed the typical basophilic inclusions of viruses of the group. Five out of twelve mice inoculated intracerebrally with the yolk sac material died between the fifth and seventh day after inoculation. Smears from these brains stained by Macchiavello's method showed the characteristic elementary bodies of psittacosis group viruses.

The six ravens *(Corvus coronoides)*, two noisy miners *(Myzantha melanocephala)* and the single specimens of three species of raptorial bird, were negative.

Domestic and aviary-bred birds.

The domestic and aviary-bred birds reported in this series have not been collected systematically, but, except for the pigeons and one lot of roosters, have been received from aviculturalists who had been disturbed by excessive deaths. The roosters tested were six brought in for preparation of antibodies, and nine belonging to an aviculturalist who had psittacosis among his parrots. The pigeons were brought in during an attempt to find a psittacosis-free strain for use in preparing antibodies.

TABLE 2.

Domestic and Aviary Birds.

Species	1951 Number tested	1951 Number positive	1952 Number tested	1952 Number positive
Homing Pigeons	43	26*	—	—
Roosters	6†	0	9†	0
Muscovy Duck *(Cairnina moschata)*	—	—	1	0
Budgerigars *(Melopsittacicus undulatus)*	—	—	9	3‡
Pileated Parrot *(Purpureicephalus spurius)*	—	—	1	1
Finches *(Various exotic)*	—	—	8	0
	49	26	28	4 (at least)

* Mainly diagnosed serologically, but in selected cases strains of virus were isolated.

† Examined by the indirect complement fixation test on serum specimens only.

‡ One of these strains was isolated from a pool of spleens from two birds and assumes that only one was infected.

The pigeons were bled on arrival in the laboratory, and placed in separate cages. When the results of the serological tests were known the positive birds were discarded (except for four which were used for the isolation of strains of virus). The birds, which were serologically negative on arrival were placed in pairs in cages and retested after a month. Those positive on either occasion are included as infected. We have been able to confirm the finding of Karrer, Meyer and Eddie (1950) that certain pigeon sera which do not fix complement in the presence of psittacosis antigen, are capable of inhibiting the subsequent fixation of complement by a known positive serum. We have also confirmed their finding that pigeon sera which show a prozone in the direct complement fixation test give a clear-cut positive reaction in the indirect complement fixation test. We did not find any uninfected stocks of pigeons, and 26 out of the 43 birds examined gave evidence of infection.

The cockerels were from two sources. The first six from a large poultry farm with no evidence of disease in the flock. The second from the poultry of an aviculturalist who had psittacosis among his parrots. In neither case did we find evidence of infection from the sera tested.

The other birds were from aviaries. Five budgerigars were from an apparently healthy aviary. None showed infection. Four were from an aviary with disease—at least three of these had psittacosis virus in the spleen. The pileated parrot *(Purpuriercephalus spurius)* was received dead from another aviary with an outbreak of disease. Psittacosis virus was isolated from this bird.

The finches were from aviaries affected with epizootics apparently not due to psittacosis virus, and in one case certainly due to pasteurellosis.

It is, perhaps, of interest to record that the most recent case of human psittacosis which we have seen was in a man who had recently bought the house of an aviculturalist and developed the disease 10 days after clearing up the yard in which the birds had been kept.

DISCUSSION.

The first investigation of the infection of wild Australian parrots with psittacosis was made by Burnet during 1934. He found in wild red-rumped parrots from Victoria an even higher infection rate than we have found in this investigation. Seventeen out of 45 birds tested were regarded as positive.

Among rosellas, however, he did not find any infection in the wild birds. He did not examine any wild Adelaide rosellas, but did examine 37 of the very closely related crimson rosella. (These two birds are now commonly regarded as geographical races of the same species.) Among 45 wild galahs, the only positive was from a single bird from Brisbane, Queensland.

In the 1938 epizootic King parrots *(Aprosmictus scapularis)* and crimson rosellas were the most heavily hit. The former had not been examined in 1934, but the latter had shown no infection, and it may well be that their susceptibility to the disease resulted from an absence of active immunity from infection in the nest. The same might apply to the Eastern rosellas, which were the most severely hit in the epizootic around Launceston, Tasmania.

If we are correct in our belief that the cause of the deaths of parrots near Adelaide in January, 1939, reported by Mr. Bellchambers, was psittacosis, possibly the Adelaide rosellas were also little infected at the time, and the high rate of infection which we record may have developed during the last 13 years.

On the other hand, if this is so, we have no explanation for the failure of the galahs to suffer in the same way, for they are extremely common, very gregarious, often live in the same country as heavily infected species and may have fairly close contact with them.

Further, the occurrence of fatal psittacosis in tame galahs (Burnet, 1935) does not suggest that they have an unusually high resistance to the disease.

The low rate of infection among wild non-psittacine land birds examined in this State contrasts with the widespread infection of *Laridae* recorded from the United States and Great Britain (Pollard, 1947; Miles and Shrivastav, 1951) and the infection of fulmars *(Fulmaris glacialis)* in the Faroe Islands (Haagen and Mauer, 1938; Rasmussen, 1938; Miles and Shrivastav, 1951). It is, however, likely that if a strictly comparable group of non-psittacine birds had been tested here as elsewhere, we also might have found a high rate of infection.

It is also interesting to compare the high rate of infection among homing pigeons in this State, which is not greatly different from that in other parts of the world, with our failure to demonstrate infection either in the wild, although introduced, Indian dove *(Streptopelia suratensis)* which lives mainly in centres of population, or in the crested pigeon *(Ocyphaps lophotes)* which has in recent years become very common throughout the settled areas.

Of our results in domestic and aviary birds, one can only say that the infection rate among pigeons is similar to that found elsewhere, that infection among domestic fowls is not universal and that the usual high rate of infection among aviary psittacines is present in this State.

SUMMARY.

A high rate of infection with psittacosis among red-rump parrots *(Psephotus haematonatus)* and Adelaide rosellas *(Platycercus adelaidae)* in the settled areas of South Australia is recorded.

Virus was not isolated from galahs *(Kakatao roseicapilla)* in the same areas.

A strain of virus with low mouse virulence was isolated from a pool of sparrow *(Passer domesticus)* spleens, but infection did not appear to be common.

Virus was not isolated from white-backed magpies *(Gymnorhina hypoleuca)*, crested pigeons *(Ocyphaps lophotes)*, Indian doves *(Streptopelia suratensis)* or starlings *(Sturnus vulgaris)*. Very small groups or single specimens from other species were also negative.

Twenty-six out of forty-three homing pigeons examined gave evidence of infection, and infection of aviary-bred psittacines was common.

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TRANSMISSION OF MURRAY VALLEY ENCEPHALITIS VIRUS BY MOSQUITOES

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The 1951 outbreak of Murray Valley encephalitis (MVE) had all the epidemiological characteristics of a mosquito-borne epidemic (Anderson *et al.*, 1952). With the recognition (French, 1952) that the virus responsible was a typical member of the group which includes St. Louis and Japanese B encephalitis viruses which are both known to be mosquito transmitted, it became virtually certain that this was also the natural mode of spread of MVE.

Attempts to isolate virus from mosquitoes caught in the Mildura area the following summer were fruitless (Reeves *et al.*, 1953), and it was concluded that the virus was no longer present in this region which had been heavily involved the previous year. In the absence of direct evidence of the identity of the vector or vectors concerned, the present work was undertaken to determine the species of Australian mosquitoes which were capable of transmitting the virus under laboratory conditions.

It was shown by Hammon and Reeves (1943) that nine species belonging to the genera *Culex, Aedes* and *Culiseta* were capable of transmitting the virus of St. Louis encephalitis to chickens, and the same workers (1946) have shown that seven species of culicine mosquitoes would transmit the virus of Japanese B encephalitis to chickens. In the case of both viruses, it was found that transmission occurred only after an interval of approximately six days from the infective blood meal.

Evidence is presented in this paper that eleven Australian species of culicine mosquito can become infected with MVE virus. Four of these have been studied in more detail, particularly *Culex annulirostris,* which was regarded on circumstantial evidence as a probable major vector in the 1951 epidemic (Anderson and Eagle, 1953; Burnet, 1952). The only anopheline mosquito studied, *Anopheles annulipes,* on the contrary failed consistently to become infected.

It was shown that *C. annulirostris* and *Aedes vigilax* can be infected by feeding on chickens in the viraemic phase of infection, and from 7 to 41 days

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

later transfer infection by feeding on normal chickens. The changes in titre of virus that can be isolated from mosquitoes at varying times after feeding indicate that a true biological cycle with multiplication of virus in the insect is involved.

Throughout this work, titration of virus in mosquitoes or other infective material has been by the chorioallantoic method. It is believed that this is the first occasion on which such a method has been used for a project of this type.

Methods and Materials.

Most mosquitoes used in these experiments were obtained wild from the Albury and Mildura regions between December, 1952, and February, 1953. Albury and Mildura, both of which are large towns on the Murray River, each provided several human cases of Murray Valley encephalitis during the epidemic of 1951. Since the epidemic there has been no evidence of virus in either district (Anderson and Eagle, 1953). Large numbers of adult mosquitoes were obtained from fowl houses or similar shelters during the daytime; others were captured when feeding on humans or cows during the evening. Several hundred *Aedes vittiger* adults were collected from a recently flooded pasture on two occasions during December, 1952. *Aedes occidentalis* and *A. notoscriptus* were obtained from two sources near Melbourne. Larvae of some species were obtained on several occasions, and were bred through in the laboratory.

The identifications of adult mosquitoes and larvae were made by Mr. N. Dobrotworsky of the Zoology Department, University of Melbourne, who assisted the author in the collection of specimens in the field.

The adult mosquitoes were held in square cages 8″ × 8″ × 8″ at a temperature of 60° to 65° F. in a portion of the building sheltered from direct sunlight. High humidity in the cages was attained by the daily application of damp towels. The mosquitoes were fed on an aqueous sugar solution.

After infection, all mosquitoes were held in similar cages in an insectary at 80° F. and relative humidity approximately 70 p.c. Each cage was provided with a jar of water, and the mosquitoes were fed on raisins. Under these conditions, mosquitoes have survived at least five weeks.

Female mosquitoes were infected by feeding a mixture of stock MVE virus, seed number 77685, diluted 10^{-3} in 5 p.c. rabbit serum saline with an equal quantity of defibrinated rabbit blood. 1 ml. of this suspension contained approximately 10^6 egg infective doses (EID). 1 EID is the smallest amount of virus producing at least one pock when tested on the chorioallantois of 11-day chick embryos. The mosquitoes were put in 4″ × 1″ glass tubes, the mouth of the tube was covered with gauze, and the mixture was applied to a small piece of cotton wool placed on the gauze. In some instances, a 5 p.c. sucrose solution in tap water was also added to the blood-virus mixture. Preliminary starvation of mosquitoes did not increase the percentage which fed on the suspension.

After feeding, all engorged mosquitoes were liberated into a cage. A pool of one or two mosquitoes was removed immediately after feeding, which will be referred to subsequently as "zero" time, and at intervals of one or more days, for estimation of virus content.

Culex pipiens australicus (Dobrotworsky and Drummond. 1953) would not feed on any mixture which contained blood. It was successfully fed on a mixture of stock virus dilution and 5 p.c. sucrose.

Titration of virus in mosquitoes. Pools of one or more mosquitoes were removed from the cage and killed with chloroform vapour. The mosquitoes were then sealed in sterile glass tubes, which were immediately frozen in a dry ice-alcohol mixture. Later the tubes were transferred to a solid CO_2 refrigerator and were stored at —70° C.

After thawing, the mosquitoes were ground with alundum in a sterile mortar, and an extract was made with 1 ml. of 5 p.c. rabbit serum saline. This rabbit serum contained no antibody to MVE virus. The extract was centrifuged at 2,000 r.p.m. for 2 to 3 minutes in an angle centrifuge. Portion of the clear supernatant was sealed in ampoules and stored at —70° C. Antibiotic was added to the remaining supernatant to give a concentration of penicillin 500 units per ml. and streptomycin 5 mg. per ml., and 0·05 ml. aliquots of the mixture were inoculated on the chorioallantois of 11-day chick embryos in groups of four.

The eggs were incubated at 37° C. for 44 hours, after which the number of specific MVE virus pocks was determined. If virus was found to be present in the mosquito extract, this was thawed and serial tenfold dilutions were made in 5 p.c. rabbit serum saline. 0·05 ml. aliquots of these were inoculated on the chorioallantoic membranes of four eggs. It is assumed that the number of pocks is equivalent to the number of egg infective doses (EID) in the inoculum. The term "titre," unless otherwise qualified, indicates the number of EID of MVE virus per mosquito pool, when used in this paper.

Feeding of mosquitoes on chickens. Chickens less than one week old were used in this series of experiments since they have been shown to develop a high titre viraemia within the first 48 hours after subcutaneous inoculation of small amounts of MVE virus (McLean, 1953). The chickens were strapped to a rack, an area of down was removed from the flank with scissors, and the mosquitoes, contained in a 2" × 1" glass tube, whose mouth was covered with cotton netting, were placed on this bare area. After several minutes or hours in the darkened insectary, the mosquitoes fed on the chicken. These mosquitoes were either killed immediately, or were separated from those which did not become engorged, after placing in a square cage. The chickens thereafter were allowed to run in the brooder, and were bled from the wing vein 24 and 48 hours subsequently.

Blood obtained from the young chickens was mixed with an equal quantity of physiological saline containing 2 p.c. sodium citrate, sealed in ampoules and stored at -70° C. Later the material was thawed, diluted 10^{-1} with 5 p.c. rabbit serum saline and inoculated on the chorioallantois of 11-day chick embryos. The number of EID of virus per ml. of blood was determined from the pock count in a manner analogous to that used in the mosquito titrations. The criterion of transmission of infection from mosquito to chicken was the development of viraemia either 24 or 48 hours after biting.

Dissection of mosquitoes. Pools of one or two mosquitoes were killed with chloroform. The legs and wings were removed by means of sterile mounted needles. The mosquitoes were then floated in one drop of 5 p.c. rabbit serum saline on a sterile glass slide. The abdomen was detached from the thorax. Steadying the thorax with one needle, the head was gently pulled, and usually the salivary glands remained attached to it. Each anatomical unit of the specimen was ground separately and extracted with rabbit serum saline, and a separate titration of each extract was performed.

RESULTS.

Range of species capable of carrying MVE virus 10 days after feeding on virus in defibrinated blood.

Preliminary experiments which were conducted with *Aedes occidentalis* and *Culex molestus* indicated that both species carried much virus on the sixth and subsequent days after feeding on MVE virus suspended in defibrinated rabbit blood.

Ten days after feeding on MVE virus suspension, pools of one or two mosquitoes of each species were examined on the chorioallantois for presence

of virus. Eleven species which belonged to the genera *Culex* and *Aedes* were found to be capable of carrying MVE virus at a titre greater than $10^{4\cdot0}$ EID per mosquito. The mosquitoes were *Culex (Culex) annulirostris* Skuse; *Culex (Culex) fatigans* Wied.; *Culex (Culex) globocoxitus* Dobr.; *Culex (Culex) pipiens australicus* Dobr. and Drumm.; *Culex (Culex) pipiens molestus* Forskal.; *Aedes (Ochlerotatus) camptorhynchus* Thoms.; *Aedes (Finlaya) occidentalis* Skuse; *Aedes (Finlaya) notoscriptus* Skuse; *Aedes (Ochlerotatus) theobaldi* Tayl.; *Aedes (Ochlerotatus) vigilax* Skuse; *Aedes (Ochlerotatus) vittiger* Skuse.

Eight separate batches of *Anopheles (Myzomia) annulipes* Walk. were fed virus suspension. The zero titre ranged between $10^{2\cdot1}$ and $10^{2\cdot8}$ EID per mosquito. Two pools each of two mosquitoes were examined two days after feeding, four pools each comprising one, two or three mosquitoes were examined on the sixth day, and five pools each being composed of from one to ten mosquitoes were examined on the tenth day. In no case was virus detected in any pool. It is concluded that *Anopheles annulipes* is incapable of carrying MVE virus under experimental conditions. This species is the only one examined in this laboratory which did not carry the virus.

Evidence that MVE virus undergoes a cycle of multiplication in several species of mosquito.

Several batches of mosquitoes of each species were fed MVE virus in defibrinated blood on different days. Immediately after removal of mosquitoes which did not feed, one engorged mosquito was killed and examined for virus content. At various days after feeding, pools of one or two mosquitoes were removed for estimation of virus content.

Titres of virus in each pool of mosquitoes at daily intervals after feeding on virus suspension are recorded in Table 1 and Fig. 1.

TABLE 1.

Representative titres of MVE virus in mosquitoes after infective blood meal.

Species	Log EID of MVE virus per mosquito pool at intervals after feeding						
	Zero	1 day	2 days	4 days	6 days	10 days	14 days
C. annulirostris	2·3	1·6	0	—	3·6	—	4·9*
C. fatigans	2·9	0	0	0	—	—	5·0*
A. vigilax	2·8	1·6	2·5	2·9	3·8	3·9	—
A. vittiger	3·1	—	2·8	—	5·7	5·0	—
An. annulipes	2·1	—	0	—	0	0	—

* These mosquitoes infected chickens by biting. — Not tested.

From a titre usually between $10^{2\cdot5}$ and $10^{3\cdot0}$ EID per mosquito at zero time, the titre frequently fell below $10^{1\cdot3}$ EID on the second day so that it could not be detected by the techniques employed in this work. The titre was usually

rising by the fourth day, and reached a level approximately 100 times higher than the titre at zero on the sixth and subsequent days. Virus has been recovered from *A. occidentalis* at 38 days and *C. annulirostris* 41 days after the infective blood meal. Mosquitoes examined more than six days after a meal nearly always contained virus at or above a titre $10^{4\cdot0}$ EID per mosquito.

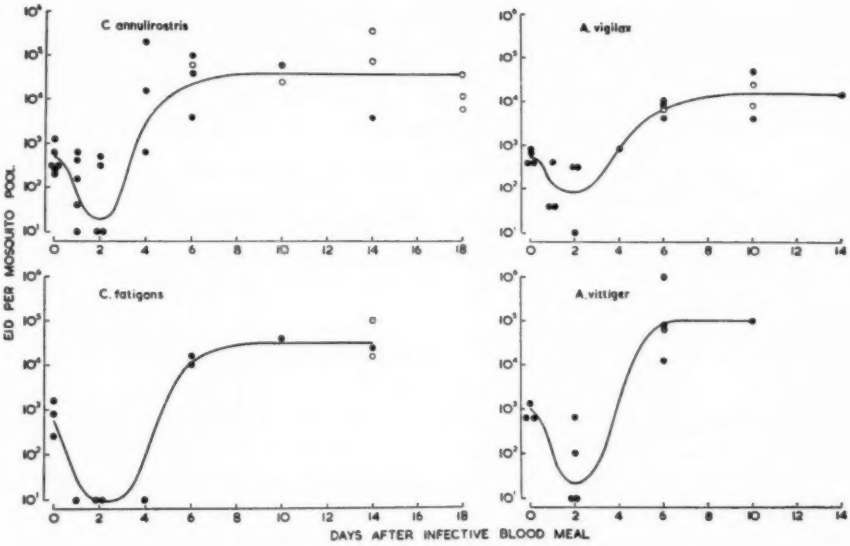

Fig. 1. MVE virus content of mosquitoes at intervals after feeding. ○ These mosquitoes transmitted virus by biting chickens.

In some instances a low positive titre was recorded in mosquitoes killed on the second day. This was observed more frequently if sugar had been added to the suspension of blood and virus. In no case, however, was the titre at this time greater than that observed immediately after feeding. On the first day after the infective blood meal, virus was often recovered from the mosquitoes, especially if the gut were filled with blood which was seen to be incompletely digested. In this case also, the titre never exceeded the titre at zero time.

In attempt to determine the site of multiplication of MVE virus, mosquitoes in pools of two were killed at daily intervals after the infective blood meal and the anatomical units: head, together with salivary glands, thorax, abdomen, legs, were examined separately for virus content. The results are expressed in Table 2.

During the first day after feeding on infected blood, virus was isolated only from the abdomen, and the gut was seen to be filled with blood. On the second

day, no virus could be detected in any portion of the mosquito, including the legs. The titre in the abdomen on the fourth and subsequent days was significantly higher than the initial titre in the abdomen, and virus also appeared in other parts of the mosquito. These observations are consistent with the hypothesis that the virus undergoes a cycle of multiplication in the mosquito. Presumably the virus enters the cells lining the walls of the stomach and midgut, undergoes multiplication within them, becomes liberated into the haemolymph as shown by the presence of virus in suspensions of legs, and thereby reaches all other parts of the body, including the salivary glands.

TABLE 2.

Virus content of anatomical units of C. annulirostris.

Days after feeding	Log EID of MVE virus in each anatomical unit			
	Head and salivary glands	Thorax	Abdomen	Legs
zero	0, 0, 0	0, 0	2·8, 2·0, 2·5	
1	0	0	2·2	
2	0	0	0	
3	0, 2·9	0, 4·2	0, 5·1	2·9
4	2·8, 2·9, 1·6	4·9, 3·1, 2·6	5·3, 4·8, 3·9	
6 or 7	4·2, 4·2	4·6, 4·8	4·7, 4·4	3·0
21	4·3	4·7	4·2	

Four mosquitoes were dissected and relevant anatomical units were weighed. The approximate relative weights were: thorax, 50 p.c.; abdomen (not engorged), 25 p.c.; head, including salivary glands, 15 p.c.; legs and wings together, 10 p.c.

Transmission of MVE virus to chickens by mosquito bite.

Mosquitoes of several species were fed on chickens aged less than one week, at various intervals after the infective blood meal. In most cases the mosquitoes were killed and examined for virus content shortly after they had fed on the chickens. Viraemia occurring in the chickens either 24 or 48 hours after the mosquito bite was taken as evidence that transmission of infection had taken place from mosquito to chicken. The results are expressed in Table 3.

Transmission of MVE virus from mosquito to chicken regularly took place on the seventh and subsequent days after the infective blood meal. *Culex annulirostris, C. fatigans, Aedes occidentalis* and *A. vigilax* have been efficient vectors under laboratory conditions.

One *C. annulirostris* fed on a chicken 9 days after the infective blood meal. This chicken developed viraemia. The same mosquito fed on another chicken 9 days later, and the second chicken became viraemic. The same mosquito contained $10^{4\cdot1}$ EID of virus.

TABLE 3.

Transmission of MVE virus from mosquito to chicken by biting.

Species	Number of mosquitoes	Days after infective blood meal	Transmission of virus	Log EID virus in mosquito pool
C. annulirostris	2	3	0/1*	4·8†
	1 or 2	7 to 41	8/8	3·8 to 5·6
	1	9	1/1	4·1
		18	1/1	
C. fatigans	2	6	0/1	4·2
	2	13	2/2	4·2 to 5·0
A. occidentalis	2 or 5	8, 13	2/2	2·8, 3·8
A. vigilax	3	2	0/1	4·4†
	4	3	0/1	4·4†
	1 or 2	6 to 12	4/4	3·8 to 4·0

* Numerator: Number of experiments in which virus was transmitted to chickens by mosquito bite. Denominator: Number of experiments performed.

† Log EID virus 10 days after infective blood meal.

On several occasions, mosquitoes were induced to bite chickens on the second or third day after the infective blood meal, when the amount of virus in the mosquito was known to be low. In no case did transmission occur. When the same mosquitoes were fed on chickens one week later, transmission occurred (Table 4). This finding would be expected from a study of Fig. 1, and is further evidence in favour of the hypothesis that a cycle of multiplication of virus takes place within the cells of the mosquito.

TABLE 4.

Transmission of MVE virus from mosquitoes to chickens after, but not during, the eclipse phase.

Species	Number of mosquitoes	Days after infective blood meal	Tranmission of virus*	Log EID virus in mosquito pool†
A. vigilax	1	2	—	3·8
		9	+	
C. fatigans	2	6	—	4·2
		13	+	

* Determined by detection of viraemia in chicken 24 or 48 hours after mosquito bite.

† Determined after second feed on chicken.

Transmission of MVE virus to mosquitoes by feeding on viraemic chickens.

Chickens aged approximately one week were rendered viraemic by the subcutaneous injection of 0·1 ml. of MVE stock virus diluted 10^{-4} in rabbit serum saline. Forty-eight hours later several uninfected mosquitoes were induced to

bite the chickens. The mosquitoes which fed were kept in a cage in the insectary until examined for virus content. Table 5 shows that virus was recovered in high titre from the mosquitoes 7 days or more after feeding on the chickens.

TABLE 5.

MVE virus in C. annulirostris *after feeding on viraemic chicken.*

Number of mosquitoes	Viraemia in chicken	Days after feeding on chicken	Virus in mosquito pool
1		zero	† <1·3
1	* 3·3	7	4·8
2		12	4·9
3	4·2	11	3·6
1	3·7	12	4·1
1	3·4	13	5·3

* Log EID virus per ml. blood. † Log EID virus per mosquito pool.

Transmission of MVE virus from chicken to chicken by mosquito bite.

On several occasions, *C. annulirostris* and *A. vigilax* mosquitoes which fed on viraemic chickens, were induced to feed on normal chickens 10 to 14 days later. Viraemia was demonstrated in the recipient chickens 24 or 48 hours later, which showed that transmission of infection had taken place between two chickens by mosquito bite. Virus in high titre was also recovered from the mosquitoes (Table 6).

TABLE 6.

Transmission of MVE virus from viraemic chicken to normal chicken by mosquito bite.

Species	Number of mosquitoes	Viraemia in donor chicken	Days between two blood meals	Viraemia in recipient chicken 24 hrs.	48 hrs.	Virus in mosquito pool
C. annulirostris	1	* 3·4	10	* 3·5	* 4·1	† 4·7
	2	4·8	11	3·0	2·8	4·2
	1	3·0	13	<1·3	3·3	5·1
	2	3·8	14	3·3	4·0	4·8
A. vigilax	1	3·3	14	2·6	3·9	4·8

* Log EID virus per ml. blood. † Log EID virus per mosquito pool.

The effect of antibody on the recovery of virus from mosquitoes.

A. occidentalis mosquitoes six to nine days after the infective blood meal were fed serum, 0·03 ml. of which would neutralize at least 100 suckling mouse LD_{50} of MVE virus. Mosquitoes were killed within 1 hour of feeding and examined for virus content on the chorioallantois. Each of four mosquitoes

so tested contained between $10^{3.5}$ and $10^{4.3}$ EID of virus, which was identical with the amount of virus in similar mosquitoes which did not feed on antiserum. This observation is in keeping with the fact recorded by French (1952) that no neutralization of MVE by its antibody could be demonstrated by chorioallantoic inoculation.

DISCUSSION.

Transmission of MVE virus to chickens by the bite of culicine mosquitoes has been demonstrated under laboratory conditions. Thus MVE virus now fulfils all three epidemiological requirements of a member of group Arthropod-borne Viral Encephalitides (Burnet, 1952).

Culex annulirostris was selected for more extensive study for the following reasons: (1) it feeds on man and domestic fowls under natural conditions; (2) it is a major component of the mosquito population in the Mildura area from January to March (Reeves *et al.*, 1953; Dobrotworsky and McLean, 1953), and cases of Murray Valley encephalitis occurred during these months in 1951 (Anderson, 1952); (3) it is found predominantly in a narrow belt one to two miles on each side of the main water-courses, which is the same region in which a high proportion of domestic fowls and wild birds contained antibody to MVE several months after the 1951 epidemic; (4) it is the Australian analogue of *Culex tarsalis* Coq., which is known to be a vector of St. Louis encephalitis in America (Hammon and Reeves, 1942), and *C. tritaeniorhynchus*, which was a vector of Japanese B encephalitis in Okayama in 1948 (Hammon *et al.*, 1949).

C. annulirostris has transmitted virus in the laboratory in a manner strictly analagous to *C. tarsalis* and *C. tritaeniorhynchus*, but proof that it is a vector of MVE virus in Nature must await isolation of the virus from it immediately before or during an epidemic.

All eleven species of culicine mosquito which have been examined in the laboratory have been found to carry MVE virus; the single anopheline occurring in the region, *Anopheles annulipes*, is not a vector. This is in conformity with the findings of Hammon and Reeves (1943, 1946) that a wide range of culicine mosquitoes carry the viruses of St. Louis and Japanese B encephalitis for long periods.

Evidence that MVE multiplies within the cells of the mosquito, in a manner similar to the multiplication of Western equine encephalomyelitis virus in *Aedes aegypti* (Merrill and Ten Broeck, 1935), is provided by the following observations: (1) the presence of an "eclipse phase" lasting two or three days after the infective blood meal, during which "base-line" quantities or no virus could be isolated from mosquitoes; (2) amounts of virus often 100 times greater than the zero titre or base-line amount after the eclipse phase has passed; (3) mosquitoes did not transmit virus during the eclipse phase, yet the same mosquitoes transmitted virus one week later.

SUMMARY.

Eleven species of culicine mosquitoes can carry MVE virus at least 10 days after an infective blood meal. *Anopheles annulipes* failed to carry MVE virus.

Transmission of virus from mosquito to chicken by *Culex annulirostris, C. fatigans, Aedes occidentalis* and *A. vigilax* has been demonstrated.

There is a regular increase in the amount of virus between the second and the sixth day after infection, indicating that the virus undergoes a biological cycle in the mosquito.

The possible rôle of *C. annulirostris* as a vector is discussed.

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THE BEHAVIOUR OF MURRAY VALLEY ENCEPHALITIS VIRUS IN YOUNG CHICKENS

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The virus of Murray Valley encephalitis (MVE) was first isolated by chorioallantoic inoculation of a suspension of brain obtained from a patient dying of the disease (French, 1952). The virus was readily transferred to mice by intracerebral inoculation and a study of its behaviour in laboratory animals indicated a general resemblance to the viruses of Japanese B encephalitis and St. Louis encephalitis. This was strengthened by French's finding of a fairly close serological relation to Japanese B virus. More detailed study of a strain of MVE virus by American workers (Paterson *et al.*, 1952) showed that it represented a fourth distinct member of the group which already contained St. Louis, Japanese B, and West Nile viruses.

Combined field and laboratory investigations (Anderson *et al.*, 1952; Anderson, 1953) of MVE have made it virtually certain that wild birds constitute an important natural reservoir of virus from which it is transferred to other potential hosts by mosquito vectors. Serological surveys indicated that a large proportion of domestic fowls in the endemic area had become infected (Anderson, 1952), and French (1952) had shown that chickens infected in the laboratory developed a transient viraemia with subsequent antibody production.

Hammon and Reeves' (1943, 1946) work on the infection of young chickens with St. Louis and Japanese B viruses showed that viraemia developed on the first and second days after subcutaneous inoculation with small doses of virus. It seemed desirable to carry out an essentially similar study with MVE virus (1) as a necessary aid to the interpretation of the findings of avian infection in the field, and (2) as a preliminary to laboratory studies of the transmission of virus by Australian mosquitoes.

The present paper shows that an easily demonstrable viraemia is produced in chickens of three different ages, by very minute amounts of virus inoculated subcutaneously. This test, in fact, can detect one LD_{50} as determined by the standard method of intraperitoneal inoculation of suckling mice.

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Methods and Materials.

Day-old male chickens, incubator hatched, were supplied by one poultry farmer from an outer suburb of Melbourne, an area where MVE is not endemic. The parent hens were hatched and reared in the same locality. The young chickens were reared in brooders in an isolation block at the Hall Institute until inoculated with MVE virus. Thereafter they were kept in other brooders for at least three weeks.

MVE virus was used in the form of an emulsion of mouse brain in rabbit serum saline. The virus was in the fifth mouse brain passage (seed number 73775) and was maintained as stock virus in a dry ice cabinet at —70° C. The 50 p.c. lethal dose of this seed inoculated by the intraperitoneal route into suckling mice 5 to 8 days old was determined. It was 0·03 ml. of a $10^{-5\cdot6}$ dilution. Throughout the current study, unless otherwise stated, this was the only stock preparation of MVE virus used.

During the preparation and holding of dilutions of virus, the whole system was maintained at 4° C. in an ice bath or refrigerator.

Rabbit serum saline. Serum was derived from two rabbits known not to be carrying neutralizing antibody to MVE virus. The fresh serum was heated at 56° C. for 30 minutes and then diluted in 0·9 p.c. saline solution to a strength of 10 p.c. when used as a diluent for mouse inoculations, or to a strength of 5 p.c. when used as a diluent for egg inoculations with MVE virus. It was found that if the 5 p.c. solution were used in mouse titrations, the end point was not clear cut, but this was corrected by the use of a 10 p.c. solution. On the contrary, in chorioallantoic titrations of MVE virus, the end point was sharp whether a 5 p.c. or a 10 p.c. solution of rabbit serum saline was used as diluent.

Antibiotics were added to all suspensions of infective material immediately prior to their inoculation into eggs or mice, to give a final concentration of penicillin 500 units per ml. and streptomycin 5 mg. per ml.

Chicken blood was obtained during life from the wing vein, or from the heart immediately after death.

Blood obtained for estimation of virus content was mixed with an equal quantity of physiological saline containing 2 p.c. sodium citrate, and stored in sealed ampoules in a dry ice cabinet at —70° C. Before inoculation into chick embryos, the material was thawed at room temperature and diluted 1 in 10 in 5 p.c. rabbit serum saline.

Chick tissues were ground with sterile alundum, extracted with 5 p.c. rabbit serum saline to give approximately a 10 p.c. suspension of tissue, and spun in an angle centrifuge at 2,000 r.p.m. for 3 to 5 minutes. The clear supernatant was stored in sealed ampoules at —70° C.

Titration of virus on the chorioallantois. The standard technique described by Beveridge and Burnet (1946) was followed. Embryos were inoculated after 11 days' incubation at 39° C., and after inoculation were further incubated at 37° C. for 44 hours. Three or four eggs were inoculated with 0·05 ml. aliquots of each dilution tested.

The number of pocks on the membranes was then determined. It was assumed that the number of pocks was equivalent to the number of egg infective doses (EID_{50}) in the inoculum.

Sera were stored at —20° C. in a mechanical deep freeze. Immediately before use the sera were thawed at room temperature and then heated at 56° C. for 30 minutes.

Titration of infectivity of MVE virus in suckling mice. Families of six suckling mice, each five to eight days old, were inoculated intraperitoneally with 0·03 ml. of serial tenfold dilutions of stock MVE virus. Antibiotic was included in each dilution. Mice which became infected developed signs of acute encephalitis after an incubation period of six or seven days and usually died within the succeeding 24 hours. All surviving mice were observed for 14 days. The 50 p.c. lethal end point was determined by the method of Reed and Muench (1938).

Neutralizing antibody was detected in sera by a similar method to that used in infectivity titrations. A volume of 0·15 ml. of serum was mixed with 0·2 ml. of dilution of stock MVE virus. This dilution contained antibiotic in the concentration mentioned above. The mixture was held at 4° C. for 30 to 90 minutes, after which 0·03 ml. aliquots were inoculated intraperitoneally into the mice. Specific deaths in the absence of antibody occurred between six and eight days after inoculation. The survivors were observed until the fourteenth day.

Dosage. In this work the suckling mouse inoculated intraperitoneally has been used as the standard of dosage. Therefore the term LD_{50}, when used in this paper, shall signify the number of suckling mouse 50 p.c. lethal doses of virus.

EXPERIMENTAL.

Titration of infectivity of MVE in chickens.

Two-day-old chickens inoculated subcutaneously. Two-day-old chickens in groups of five were injected subcutaneously in the left pectoral region with 0·1 ml. of serial tenfold dilutions of stock MVE virus. Forty-eight hours later the chickens were bled and the titre of virus in each sample was determined. No specific clinical signs believed to be due to MVE virus were observed during the subsequent 14 days.

The criterion of infection of chickens was the development of viraemia 48 hours after inoculation.

A parallel titration of the same stock of virus was carried out in suckling mice.

TABLE 1.

ID_{50} of MVE virus for two-day-old chickens inoculated subcutaneously compared with LD_{50} for suckling mice inoculated intraperitoneally.

Inoculum	Viraemia in chickens (0·1 ml. injection)	Deaths in mice (0·03 ml. injection)
10^{-7}	5/5*	18/18†
10^{-8}	6/10	16/18
10^{-9}	8/15	3/18
10^{-10}	0/5	1/6

* Numerator: Number of chickens which had viraemia 2 days after inoculation of 0·1 ml. Denominator: Number of chickens tested.

† Numerator: Number of mice which died with acute encephalitis after inoculation of 0·03 ml. Denominator: Number of mice tested.

ID_{50} for two-day-old chickens: 0·1 ml. of a $10^{-8\cdot8}$ dilution.

LD_{50} for suckling mice: 0·03 ml. of a $10^{-8\cdot6}$ dilution.

The results obtained in three separate parallel titrations of the one seed in chickens and mice are combined to form Table 1. The ID_{50} for two-day-old chickens was 0·1 ml. of a $10^{-8\cdot8}$ dilution of the seed tested, and the ID_{50} for suckling mice was 0·03 ml. of a $10^{-8\cdot6}$ dilution of the same seed.

Four-day-old chickens fed virus. Groups of five chickens, each four days old, were fed virus by instilling 0·2 ml. of virus dilution on the back of each tongue. Each chick was assumed to have ingested 0·1 ml. of material. The

chickens were bled two days later, and the blood examined for virus content. The proportion of chickens developing viraemia is shown in Table 2. The infectivity end point is thus approximately 10^{-2}, or 10^{7} LD_{50}.

TABLE 2.

ID_{50} of MVE virus for four-day-old chicks fed virus.

Inoculum	Viraemia in chickens
10^{-2}	2/5*
10^{-3}	1/5
10^{-4}	0/4

* Numerator: Number of chicks which had viraemia 2 days after inoculation. Denominator: Number of chicks tested.

ID_{50} of four-day-old chicks fed virus orally is approximately 10^{-3} for a 0·1 ml. inoculum.

TABLE 3.

ID_{50} of MVE virus for 28-day-old chicks inoculated subcutaneously.

Inoculum	Viraemia in chicks
10^{-6}	4/4*
10^{-7}	3/4
10^{-8}	0/4

* Numerator: Number of chicks which had viraemia 48 hours after inoculation. Denominator: Number of chicks tested.

ID_{50} of 28-day-old chicks: $10^{-7\cdot3}$ for a 0·1 ml. inoculum.

Twenty-eight-day-old chickens inoculated subcutaneously. Groups of four chicks, each 28 days old, received 0·1 ml. of virus dilution subcutaneously in the left pectoral region. The proportion of chicks developing viraemia at 48 hours is indicated in Table 3.

From these figures, the ID_{50} of MVE virus in 28-day-old chicks is $10^{-7\cdot3}$, which is somewhat more than ten times the LD_{50} for baby mice.

The distribution of virus in chickens infected by subcutaneous route.

Groups of five chickens, each two days old, were injected subcutaneously in the left pectoral region with 0·1 ml. of a 10^{-3} dilution of MVE virus. This represents approximately 10^{6} LD_{50}. The groups were inoculated on separate occasions over a period of one month. All the members of any one group were examined for viraemia at the same time, when some of the chicks in the group were killed and the spleens examined for virus content.

TABLE 4.

Virus content of blood and spleen in two-day-old chicks after subcutaneous inoculation of 10^{6} LD_{50} of MVE virus.

Days after inoculation	1	2	3	4
Egg-infective doses of virus per ml. of *blood*	$10^{6\cdot6}$, $10^{4\cdot6}$, $10^{4\cdot6}$ $10^{3\cdot3}$, 0	$10^{7\cdot0}$, $10^{6\cdot9}$, $10^{4\cdot9}$ $10^{4\cdot6}$, $10^{3\cdot9}$	$10^{7\cdot7}$, $10^{5\cdot8}$, $10^{5\cdot5}$ $10^{5\cdot5}$, $10^{3\cdot0}$	$10^{7\cdot6}$, $10^{7\cdot3}$, $10^{7\cdot1}$ $10^{5\cdot6}$, $10^{5\cdot4}$
Geometric mean	$10^{3\cdot9}$	$10^{5\cdot4}$	$10^{6\cdot1}$	$10^{6\cdot6}$
Egg-infective doses of virus per gm. of *spleen*	NT	NT	$10^{5\cdot5}$, $10^{5\cdot5}$	$10^{5\cdot9}$, $10^{5\cdot5}$
Geometric mean	—	—	$10^{5\cdot5}$	$10^{5\cdot8}$

TABLE 4 (continued).

Days after inoculation	5	8	13	27
Egg-infective doses of virus per ml. of *blood*	$10^{6\cdot6}$, $10^{6\cdot1}$ $10^{2\cdot7}$, $10^{1\cdot8}$	$10^{5\cdot3}$, $10^{2\cdot0}$, 0 0, 0	0, 0, 0 0, 0	0, 0, 0 0, 0
Geometric mean	$10^{4\cdot0}$	$10^{1\cdot4}$	0	0
Egg-infective doses of virus per gm. of *spleen*	$10^{5\cdot8}$, $10^{5\cdot3}$ $10^{5\cdot1}$, $10^{4\cdot8}$	$10^{5\cdot4}$, $10^{5\cdot4}$, $10^{2\cdot5}$ 0, 0	0, 0, 0 0, 0	0, 0, 0 0, 0
Geometric mean	$10^{5\cdot0}$	$10^{2\cdot6}$	0	0

Table 4 and Fig. 1 indicate that a high level of viraemia is established within the first 24 hours after inoculation, and persists at or above this level until the fifth day. The splenic content evidently runs parallel with that of the blood after the first 48 hours following inoculation. Virus was detected occasionally in blood and spleen on the eighth day, but could not be recovered on the thirteenth or subsequent days. Each of the chickens killed five days or more after inoculation had been shown to have had a viraemia at 3 days.

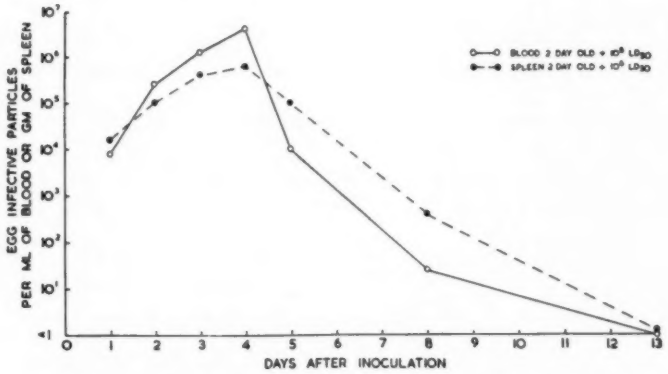
EGG INFECTIVE PARTICLES PER ML. OF BLOOD OR GM OF SPLEEN
DAYS AFTER INOCULATION
BLOOD 2 DAY OLD + 10⁶ LD50
SPLEEN 2 DAY OLD + 10⁶ LD50

Fig. 1. Virus content of blood and spleen in two-day-old chickens after subcutaneous inoculation of 10^6 LD_{50} of MVE virus.

When a similar experiment was performed using stock virus which had undergone two further mouse brain passages than seed 73775, similar results were obtained. However, the titres of virus in blood and spleen were in general 1 to 2 log EID_{50} lower than those recorded in Table 4, and virus was not recovered from blood or spleen on the sixth or subsequent days after inoculation.

In attempts to determine the site of multiplication of virus in the young chicken, eight chicks, each two days old, were inoculated subcutaneously in the left pectoral region with approximately 10^6 LD_{50} of MVE virus, seed number 74439. Groups of two birds were killed immediately afterwards, and at 6, 24 and 48 hours. Site of inoculation, blood, spleen, liver and brain were examined for virus content. The "site of inoculation" was the oedematous area of subcutaneous tissue overlying the pectoralis major muscle, and was the area into which the suspension had been injected. The skin was removed, and then the site of inoculation was excised and extracted with rabbit serum saline. Often some muscle was included in the mass of tissue excised. The results are shown in Table 5.

TABLE 5.

Titres of MVE virus in several organs of two-day-old chicks following subcutaneous inoculation of 10^6 EID_{50}.

Organ	Time after inoculation							
	10 minutes		6 hours		24 hours		48 hours	
Site of inoculation	$10^{5\cdot1}$	$10^{5\cdot5}$*	Z	$10^{2\cdot7}$	$10^{5\cdot7}$	$10^{2\cdot7}$	$10^{3\cdot8}$	$10^{3\cdot8}$
Blood	—	—	Z	Z	$10^{1\cdot7}$	$10^{2\cdot1}$	$10^{2\cdot5}$	$10^{2\cdot6}$
Spleen	—	—	Z	Z	$10^{5\cdot6}$	$10^{2\cdot6}$	$10^{2\cdot5}$	$10^{2\cdot4}$
Liver	—	—	Z	Z	$10^{2\cdot8}$	$10^{2\cdot5}$	Z	$10^{2\cdot9}$
Brain	—	—	Z	Z	$10^{3\cdot5}$	Z	$10^{2\cdot5}$	$10^{2\cdot5}$

* All titres expressed as number of egg-infective doses of virus per gm. of tissue or per ml. of blood.

— = not tested. Z = Titre $<10^{2\cdot3}$ EID_{50} per ml. of blood or gm. of tissue.

In a further experiment, pairs of chickens were killed at the same time intervals, 10 minutes, 4 hours, 8 hours and 24 hours after inoculation. Virus could not be recovered from any tissue examined at 4 hours. At 8 hours virus was demonstrated only at the site of inoculation, and at 24 hours it was recovered from each tissue examined.

The findings are compatible with the hypothesis that the virus may undergo multiplication in the endothelial cells lining the blood vessels throughout the body. Further studies on this problem will be reported subsequently.

Viraemia in older chicks.

Chickens 4 days old and 28 days old in groups of 6 and 9 respectively received 10^3 LD_{50} of MVE virus subcutaneously in the left pectoral region. Blood taken at 24 and 48 hours after inoculation was examined for virus content. The figures shown in Table 6 indicate that older chickens also regularly develop a viraemia 48 hours after inoculation. The figures show that the titre of virus is less in older chickens than in chickens under one week old. The older chickens showed no signs of illness during the subsequent 14 days.

TABLE 6.

Comparison of viraemia occurring in chicks four days old and twenty-eight days old after subcutaneous inoculation of 10^3 LD_{50} of MVE virus.

Age of chick	Hours after inoculation	Egg-infective doses of virus per ml. of blood									Geometric mean
4 days	24	$10^{3\cdot3}$		$10^{3\cdot5}$	$10^{3\cdot6}$	$10^{3\cdot7}$	$10^{5\cdot1}$	$10^{5\cdot7}$			$10^{4\cdot1}$
	48	$10^{3\cdot6}$		NT	$10^{3\cdot9}$	$10^{5\cdot3}$	$10^{5\cdot3}$	$10^{3\cdot3}$			$10^{4\cdot4}$
28 days	24	0	0	0	0	$10^{2\cdot7}$	$10^{2\cdot9}$	$10^{3\cdot6}$	$10^{5\cdot5}$	$10^{6\cdot3}$	$10^{2\cdot3}$
	48	$10^{3\cdot3}$	$10^{3\cdot6}$	$10^{4\cdot3}$	$10^{4\cdot3}$	$10^{4\cdot3}$	NT	$10^{3\cdot3}$	$10^{3\cdot7}$	$10^{1\cdot6}$	$10^{3\cdot3}$

NT = Not tested.

Two cockerels approximately six months old were inoculated with 10^6 LD_{50} of virus subcutaneously. Both developed a viraemia of low titre of the order 10^2 EID_{50} per ml. 24 and 48 hours subsequently, and this was followed by the appearance of neutralizing antibody a fortnight later.

Clinical disease in chickens.

Six chickens each two days old were inoculated subcutaneously with 10^6 LD_{50} of virus, seed number 74439. Two similar chickens were kept as uninoculated controls. The cloacal temperatures were recorded daily, observations of clinical signs were made daily, and blood was obtained for titration of virus content.

Three of the six inoculated chickens and one of the control chickens survived at least 14 days, and at no time did these birds exhibit any abnormal clinical signs. Two of the six inoculated chickens and one of the control chickens became ill on the eighth day after inoculation, and died within the next 24 hours. The remaining inoculated chicken became ill on the fourth day, and died on the fifth day after inoculation. All these sick chickens exhibited the same syndrome, which consisted of weakness of all four limbs, lethargy and ataxia, followed later by arching backwards of the head and aimless picking at the ground. Exactly similar signs are observed in many other non-infected chickens aged less than two weeks if the brooder had been turned off for more than six hours.

All the inoculated chickens in this experiment developed a transient viraemia between the first and fourth days after inoculation. In general, the temperatures of all the inoculated chicks did not differ by more than 1° F. from either their own pre-inoculation temperatures, or from the temperatures of the control chicks on the same day. On the fourth day the temperatures of all the inoculated chicks except one were somewhat elevated.

In each of the experiments with two-day-old chicks reported earlier in this paper, some of the chickens died between the fifth and tenth days after inoculation, but chickens which were hatched at the same time and were not inoculated also died after exhibiting the same clinical picture. The mortality rate for uninoculated chickens was approximately 25 p.c. It is therefore considered that the deaths amongst the groups of inoculated chickens were probably non-specific.

Four chickens each four weeks old were inoculated subcutaneously with 10^7 LD_{50} of MVE virus, seed number 74439. Daily observations of cloacal temperatures and clinical signs were made, and the titre of virus in the blood was determined on the first, second, fourth and sixth days.

Throughout the period of observation, which extended at least 14 days, all the chickens remained clinically normal, and their temperatures showed no wide fluctuation from the pre-inoculation level. All chickens had a viraemia at 48 hours after inoculation. Three of the four chickens were viraemic on the first day after inoculation, but none had viraemia on the fourth or sixth days.

In other experiments with four-week-old chickens reported in this paper, none was observed to exhibit any clinical abnormality which could be attributed to infection with MVE virus.

All adult fowls which have been inoculated subcutaneously with MVE virus have remained clinically normal during the period of observation, which has extended for over three months in some cases.

Serological observations.

Sera from chicks in a number of the above experiments were saved for estimation of neutralizing antibody content. In the determination of the presence of antibody in these sera, the sera were mixed with virus to give a final concentration of virus of either 50 or 500 LD_{50}. Aliquots of 0·03 ml. of the mixture were injected intraperitoneally into groups of five or six baby mice.

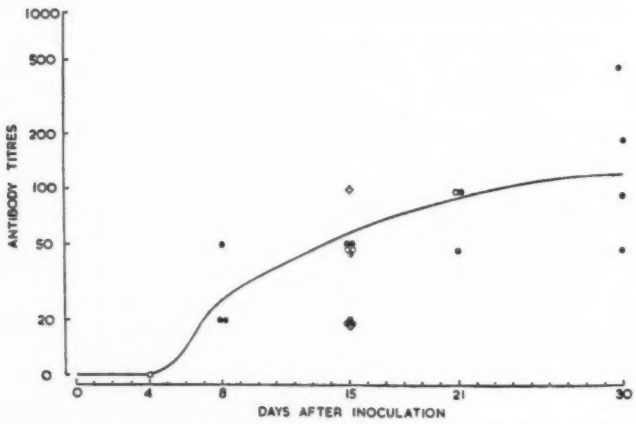

Fig. 2. Antibody titres in two-day-old chickens which were inoculated with MVE virus subcutaneously.

● 2-day-old chicken 10^6 LD_{50}
○ 2-day-old chicken 10^4 LD_{50}
-○- 2-day-old chicken 10^3 LD_{50}
◑ 2-day-old chicken 10^2 LD_{50}
◒ 2-day-old chicken 10 LD_{50}

In the interpretation of the results of mouse inoculation, the following antibody "levels" were arbitrarily assigned: 0 if 6 mice out of 6 (6/6) or 5 mice out of 6 (5/6) died between 6 and 8 days after inoculation with 50 LD_{50} plus serum, 20 if 4/6 died, 50 if 3/6 died, 100 if 2/6 died and >100 if 1/6 or 0/6 died. Using 500 LD_{50} as a final concentration of virus, the "levels" assigned were: 200 if 4/6 mice died, 500 if 3/6 died, 1,000 if 2/6 died, and >1,000 if 1/6 or 0/6 died.

TABLE 7.

Production of neutralizing antibody following subcutaneous inoculation of chickens with MVE virus.

Age of chick	Dose inoculated mouse LD_{50}	Viraemia at 48 or 72 hrs.	Antibody "levels" at intervals after inoculation*							
			Pre-inoculation	4 days	8 days	15 days	21 days	30 days	6 wks.	12 wks.	15 wks.
2 days	10^6	+			20						
"	10^6	+			20						
"	10^6	+			50						
"	10^6	+				20		50			
"	10^6	+				20		100			
"	10^6	+				20		500			
"	10^6	+				50					
"	10^6	+				50		200			
5 days	10^4	+		0		50	100				
"	10^4	+				50					
2 days	10^2	+				100					
"	10^2	+					100				
"	10^2	+				50					
"	10^1	+				20					
"	10^1	—					50				
30 days	10^6	—			0		500	1,000			100
"	10^3	+		0	20		200	200			
"	10^3	+		0		20		100			
"	10^3	not tested		0	20	100		200			50
"	10^2	not tested		0	50			200			
6 months	10^6	+	0		50	200	500	1,000	500	50	
"	10^6	+	0		20	100	200	1,000	500	100	

* See text for description. + = Viraemia was demonstrated. — = Viraemia could not be detected.

Table 7 and Figs. 2 and 3 record the results. Although the figures are approximate, they indicate that in young chickens neutralizing antibody is produced before the fifteenth day following subcutaneous inoculation with MVE virus. Older chickens and adult fowls first produced antibody between the fourth and eighth day after inoculation. It appears that the titre of antibody increases during a period of four weeks following inoculation in both age groups. Antibody was detected in two four-week-old chickens fifteen weeks after inoculation.

Two cockerels each six months old were inoculated subcutaneously with 10^6 LD_{50} of virus. Both had a viraemia on the first and second days after inoculation. Sera taken before inoculation contained no antibody to MVE. On the

ninth day after inoculation, antibody was detected at a low level. The titre rose to a maximum on the twenty-eighth day and thereafter declined, so that after twelve weeks only a minimal amount of antibody was detected.

A second injection of 10^6 LD_{50} of virus was administered subcutaneously thirteen weeks after the initial injection. Serum obtained on the sixth and thirteenth days after this injection contained neutralizing antibody at a level of 500 or more.

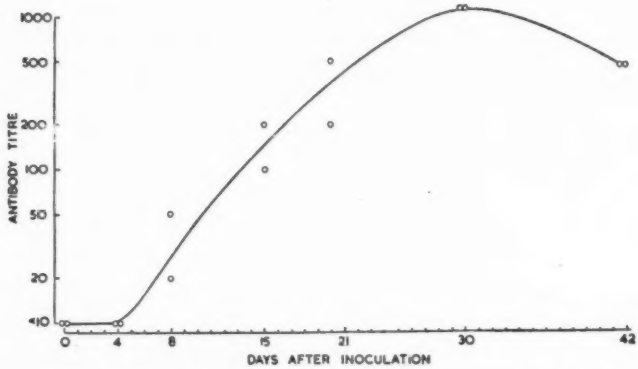

Fig. 3. Antibody titres in six-month-old roosters following subcutaneous injection with MVE virus.

Transfer of antibody through the egg.

Three hens, each approximately one year old, were inoculated with 10^6 LD_{50} of virus subcutaneously. All subsequently developed circulating antibody at low titre. Eight weeks later they received a further subcutaneous injection of 10^6 LD_{50} of virus. A rooster was allowed to run with the hens, which had now commenced to lay eggs regularly. Most eggs were incubated at 39° C., and from a high proportion, chickens were eventually obtained. Other eggs were opened shortly after being laid, and the yolk was preserved at —20° C. until titrated for antibody content. The yolk was thawed, emulsified with an equal volume of dilution of stock MVE virus, and 0·03 ml. aliquots of this mixture were injected into baby mice in groups of six.

The yolks of eggs laid before the hens were inoculated with virus were found to contain no antibody to MVE virus. The yolk of eggs laid on the sixth, thirteenth and twenty-sixth days after the second inoculation of the hens contained antibody at a high titre. The blood of the hens on the thirteenth and twenty-sixth days after the second inoculation also contained antibody at a high titre. The results are shown in Table 8.

TABLE 8.

Antibody "levels" in sera of parent hens and egg yolks laid by these hens after the hens had received two injections of 10^6 LD_{50} of MVE virus.

Hen	Source of antibody	Antibody "level" after second inoculation				
		Pre-inoculation†	6 days‡	13 days	26 days	48 days
194	serum of hen	0	NT	1,000	>1,000	1,000
	egg yolk	0	1,000	1,000	1,000	>1,000
196	serum of hen	0	NT	1,000	1,000	>1,000
	egg yolk	0	>100	1,000	1,000	>1,000
198	serum of hen	0	NT	100	1,000	1,000
	egg yolk	0	>100	>100	1,000	>1,000

† Pre-inoculation: before first inoculation of hens. ‡ Days: days after second inoculation of hens. NT: not tested.

TABLE 9.

Antibody levels in sera of chickens hatched from eggs laid by hens 194, 196, 198 between 21 and 28 days after the second inoculation.

Parent hen	Antibody levels in chickens at intervals after hatching		
	<7 days	14 days	21 days
194	500	0	0
196	>1,000	100	0
198	100	50	0

Chickens which were hatched from eggs laid by these hens between 21 and 28 days after the second inoculation were killed at weekly intervals after hatching and the antibody content of their blood was determined. The figures shown in Tables 8 and 9 provide good evidence that antibody is transferred from the parent fowl to the chicken through the egg. Antibody was not detected in the young chicken longer than two weeks after hatching.

DISCUSSION.

Viraemia in chickens regularly follows the subcutaneous injection of MVE virus. A chicken aged less than one week will develop a viraemia if injected with an amount of MVE virus approximately equal to the LD_{50} of suckling mice inoculated intraperitoneally. Whereas suckling mice regularly develop signs of acute encephalitis and die after injection of minimal doses of virus, young chickens were not observed to show clinical signs attributable to infection with MVE virus, even after a massive dose. There is a certain mortality rate, approximately 25 p.c., amongst non-infected chickens less than one week old when they are reared in a brooder, but no good evidence has been obtained that subcutaneous injection of MVE virus increases this mortality rate.

From the epidemiological standpoint, the production of a relatively high-titred and prolonged symptomless viraemia after subcutaneous injection of chickens has considerable significance. Miles (1952) found that prolonged periods of viraemia occurred in several species of wild and domestic birds after subcutaneous inoculation with the South Australian strain of MVE virus. Our own results with domestic chickens are in general agreement with those obtained by Miles. Sufficient virus is present in the blood to cause infection in mosquitoes (McLean, 1953). We conclude that any viraemic bird can act as a ready natural source of virus for any blood-sucking arthropod, for example, the mosquito. The small dose of virus capable of inducing viraemia in a young chicken or an older bird could readily be injected by the bite of one infected mosquito. Thus a cycle of infection between bird and mosquito appears possible.

The domestic fowl, by reason of its large numbers and its close proximity to human habitation, may possibly be an important reservoir of infection of humans. The mosquito vectors in this cycle of infection from fowls to humans may include *Culex annulirostris* and *C. fatigans*, both of which have been observed to bite chickens (McLean, 1953).

Observations recorded in this paper suggest that young domestic chickens, bird for bird, may be somewhat more important than older fowls as natural reservoirs of virus owing to the slightly more prolonged and the higher titred viraemia which occurs following infection. There is no evidence that virus is stored in the spleen after the viraemia has ceased.

The sera of many domestic fowls in the Mildura region seven months after the encephalitis epidemic of 1951 contained neutralizing antibody to MVE virus (Anderson *et al.*, 1952). Similarly, sera from many wild birds in the area contained antibody during the same period after the epidemic. Fowls hatched since the epidemic and exposed only to the mosquitoes of the 1951-52 summer, a summer in which there was no encephalitis epidemic in the region, were shown to be devoid of antibody to MVE (Anderson, 1952). Experiments recorded in this paper show that domestic chickens of several ages react to subcutaneous injection of MVE virus by the production of neutralizing antibody. This finding validates the use of "sentinel" fowls to detect the presence of the virus in the field during the summer months.

Antibody to MVE virus has been detected in the eggs and in newly hatched fledglings of the Little Pied Cormorant *(Phalacrocorax melanoleucos)* (Anderson, 1953) from the age of one day up to the age of 12 weeks. Our own experiments have demonstrated that maternal antibody present in the serum of the domestic hen can be transferred to the yolk and to the young chicken.

SUMMARY.

Young chickens regularly developed a viraemia following subcutaneous injection with MVE virus. The titre is sufficiently high to infect mosquitoes. Infection of chickens did not produce recognizable clinical signs.

Antibody was regularly produced following inoculation of live virus into chickens of several ages.

Experimental results are in accord with previously reported field observations on fowls following the 1951 MVE epidemic in the Murray Valley.

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THE EFFECT OF HUMAN BRONCHIAL SECRETIONS (SPUTUM) ON THE HAEMAGGLUTININ AND INFECTIVITY OF INFLUENZA VIRUS

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Since it was established that the agglutination of red cells by influenza virus and the subsequent elution of virus was essentially an enzymic process (Hirst, 1942), there has been much speculation about the rôle which the enzyme might play in infection.

Although this is still uncertain, one possible function of the enzyme, suggested by Burnet (1951), is that it might operate during the passage of virus through the film of mucus which overlies the susceptible cells of the respiratory epithelium. This layer contains mucins which will combine with virus (Himmelweit, 1948; Fazekas, 1951) and so possibly deviate it from cell receptors. Possession of an enzyme which breaks down the receptors on such soluble mucins might be of "survival value" to the virus in freeing it from the mucin and enabling it to come in contact with the infectible cell.

The action of influenza virus enzymes on soluble mucins extracted from a number of sources—human ovarian cyst, urine, meconium, sheep salivary gland, etc., and, more pertinently, human nasal mucus (Fazekas, 1952) has been extensively studied. Characteristic differences in the efficiency with which enzymes of different influenza virus strains destroy the mucin substrates have been recognized but the significance of this "inhibitor gradient" in infection is unknown.

If virus enzyme and respiratory mucin interact in the way suggested by Burnet, it might be expected that respiratory mucins would have some neutralizing action on infection by viruses with inefficient enzymes.

For this reason, the report of Rose (1950) that human bronchial mucus contains a factor, other than specific antibody, which will neutralize or inhibit the growth of virus (PR8) in eggs and mice, was of interest. It was thought that an attempt to determine the relation of this factor to the soluble mucins

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and to isolate them from other components of sputum might be of value as a preliminary approach to the problem.

The present investigation has been undertaken with these objects in view. A preliminary report on the purification of an inhibitor of haemagglutination from human sputum has already been made (Curtain, Marmion and Pye, 1953).

EXPERIMENTAL.

Materials and Methods.

Human sputum. Sputum was collected from at least 20 patients with chronic bronchitis who had a copious, but not grossly purulent or blood-stained sputum. The patients expectorated into sterile 4 oz. screw-capped jars containing 1 ml. of a saline solution of penicillin (1,000 units) and streptomycin (1 mg.). At first, 24-hour specimens from single patients were used, but later, during the isolation of the principal haemagglutinin inhibitor from sputum, samples from five to ten patients were pooled and processed. Initially, some difficulty was encountered due to growth of respiratory bacteria resistant to penicillin and streptomycin, but this was controlled by adding a few drops of purified toluene to the sputum before processing.

Normal saline. Nine gm. of A.R. sodium chloride per litre of distilled water.

Citrate saline. Normal saline containing 0·2 p.c. sodium citrate.

Calcium acetate saline (pH 6·2). 12·3 gm. sodium acetate, 1·3 ml. 2 N acetic acid, 5·0 gm. sodium chloride, 1·0 gm. calcium chloride, made up to one litre with distilled water.

Calcium borate buffer (pH 8). Sodium borate, 0·56 M.; hydrochloric acid, 0·44 M.; calcium chloride, 0·05 M.

Calcium acetate buffer (pH 3·6). Acetic acid, 0·93 M.; sodium acetate, 0·06 M.; calcium chloride, 0·02 M.

Crystalline trypsin. A sample of "Armour" crystalline trypsin was made up into a normal saline stock solution containing 50 mg. of trypsin per ml.

Trypsin inhibitor. A preparation of purified trypsin inhibitor was made from soya bean meal, using the method described by Tauber, Kershaw and Wright (1949) for lima bean.

Receptor-destroying enzyme of Vibrio cholerae *(RDE).* A highly purified preparation, made by the method of Ada and French (1950), was used.

Influenza viruses.

Viruses for use in the neutralization tests in the chick embryo were prepared by passing infected allantoic fluid at a dilution of 10^{-4} or 10^{-5} to the allantoic sac of eggs incubated for 11 days. After 48 hours' further incubation, the fresh harvested allantoic fluids were centrifuged and 0·25 ml. quantities dispensed into glass ampoules which were sealed and placed in a dry-ice cabinet.

Viruses used in the haemagglutination-inhibition test were passaged in a similar way and used in the form of infected allantoic fluids up to seven days after harvesting. These were stored at 4° C. The strains of virus were those in common use at this Institute, and accounts for their origin are given by the authors whose names follow the strain: WSE (Burnet, 1936); MEL (Burnet, 1935); CAM (Anderson, 1947); LEE (Francis, 1940); PR8 (Francis, 1934); FM1 (Rasmussen, Stokes and Smadel, 1948).

These viruses were converted to the "indicator" state using methods described by Stone (1949). When so converted they have been designated: heated WSE = H–WSE, treated MEL = T–MEL, and so on.

Neutralization tests in the chick embryo.

The neutralizing values of sputum (or extracts) was determined by mixing constant volumes of undiluted sputum with equal volumes of selected tenfold dilutions of virus and, after incubation, inoculating a portion of the mixtures sub-allantoically into chick embryos. This "constant sputum/varying virus" method gave the number of infective doses of virus neutralized by undiluted sputum and was used because of its sensitivity.

In these tests the tenfold dilutions of virus were made in 10 p.c. inactivated horse serum saline containing penicillin (100 units per ml.) and streptomycin (0·1 mg. per ml.). 0·25 ml. of virus dilution was mixed with 0·25 ml. of sputum, or horse serum saline in the controls, held at 37° C. for 30 minutes after mixing, and then transferred to a bath containing ice and water while inoculation of fertile eggs was done. 0·05 ml. of each of the sputum-virus and control mixtures was inoculated into the allantoic sac of groups of from four to eight 11- or 12-day old chick embryos using the method of Beveridge and Burnet (1946). The eggs were incubated at 36° C. for a further 48 hours and then a quantity, about 0·25 ml., of the allantoic fluid was removed and mixed with 0·25 ml. of normal saline to which 0·25 ml. of a 1 p.c. suspension of fowl cells had been added. Tubes were shaken and the pattern of the sedimented cells was read some 45 minutes later. Only tubes showing a complete "shield" of agglutinated cells were considered positive and as indicating infection in the egg. The "50 per cent. infection" end points (Reed and Muench, 1938) were calculated for both sputum and control series of eggs and the neutralization value obtained by subtraction of the $\log_{10}$ values.

Titration of inhibitor of haemagglutination by influenza virus. The method of Stone (1949) was employed. Throughout the paper the term "neutralizing" refers to the action in protecting the fertile egg from infection, while the term "inhibiting" means the action on the virus haemagglutinin *in vitro*.

High-speed centrifugation. This was carried out at 2° C., using a "Spinco" Model L ultracentrifuge. The specifications of the No. 21 and No. 30 rotors employed are given in Table 1.

TABLE 1.

Specification of centrifuge used in sputum processing.

	No. 21 rotor	No. 30 rotor
Angle of tubes to axis of rotation	18°	26°
Minimum radius of liquid from axis of rotation	6·0 cm.	5·0 cm.
Mean radius of column of liquid from axis of rotation	9·0 cm.	7·8 cm.
Maximum radius of column of liquid from axis of rotation	12·0 cm.	10·5 cm.
Capacity of each tube	94·0 ml. (10 in all)	38·5 ml. (12 in all)

Electrophoresis. Electrophoresis was carried out in a Tiselius apparatus equipped with a Philpot-Svennson scanning system (Holden, Ada and Pye, 1952). All runs were done in an 11 ml. fused Pyrex cell.

The buffers used were as follows:

Phosphate buffer. Ionic strength $\mu = 0{\cdot}1$; pH 7·0, 0·045 M. KH_2PO_4, 0·027 M. KOH.
Glycine buffer. Ionic strength $\mu = 0{\cdot}1$; pH 10·0, 0·15 M. glycine, 0·10 M. KOH.

PART I.

Isolation of inhibitors of influenza virus haemagglutination from sputum.

Preliminary treatment of sputum. The sputum samples were mixed with ten times their volume of normal saline and homogenized for 10 minutes in a Waring blendor the top of which was sealed with "Cellophane" smeared with petroleum jelly. This broke up the globlets of mucus and converted the preparation into a workable solution. The homogenized sputum was freed of bacteria and cellular debris by centrifugation at 10,000 *g* for 15 minutes in the No. 21 rotor. Material processed up to this stage will be referred to as "semi-purified supernatant." Certain batches of this supernatant prepared from sputum taken from single patients were tested for virus neutralizing activity and were also used at first to study some of the properties of the haemagglutinin inhibitors.

Ultracentrifugation of semi-purified supernatant. Semi-purified supernatant was centrifuged in the No. 30 rotor using the sucrose density gradient method of Pickels (1943) to minimize convection in the centrifuge tube. The maximum sucrose concentration used was 12 p.c. at the bottom of the tube. After centrifugation for 120 minutes at 78,000 *g*, the tubes were carefully removed from the rotor and eleven 3 ml. samples were removed from each, proceeding from the top downwards and using a Pasteur pipette with its tip ground to an angle of 45°. The long side of the pipette was held against the wall of the tube with the tip just in the meniscus as suction was applied.

Refractometric determination of the sucrose concentration in each of the 11 samples showed that little mixing had occurred during centrifugation. The sediment from the bottom of the tube was resuspended in a measured volume of normal saline and the concentration of haemagglutinin inhibitor (titrated against five haemagglutinating doses of H-LEE) was determined in the deposit and in each of the eleven layers above. It was found that 50 p.c. of the inhibitor was present in the deposit and that nearly all the rest was in the four layers just above this. The four layers were pooled, the sucrose removed by dialysis and electrophoresis carried out in the pH 7·0 phosphate buffer. The pattern obtained showed two major groups of components with mobilities of 7×10^{-5} and 5×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} respectively. When the mixture was treated with 10,000 units of purified RDE for 60 minutes at 37° C. and again analysed electrophoretically the mobility of the faster component was reduced. This effect has been shown by Pye (1953) to indicate that the substance with the decreased mobility is a substrate for RDE and for influenza virus enzyme (i.e., is an "inhibitor").

The results of this electrophoretic analysis also suggested that the inhibitor had a lower *iso*-electric point than the other components present in the mixture.

This property was used in attempts at fractionation of the material obtained from the four layers above the ultracentrifuge deposit.

Preliminary experiments with methanol fractionation at pH values 3–4 and at 0° C. proved disappointing. Very low recoveries of inhibitory activity were obtained.

Iso-electric precipitation of the inhibitor in the presence of alkaline earth cations. Curtain (1953) has shown that the solubility of the urinary mucoprotein inhibitor (Tamm and Horsfall, 1950) is markedly decreased by the presence of small concentrations of the alkaline earth cations—calcium, magnesium and barium—the latter irreversibly inactivating the inhibitor. Neutral solutions of calcium and magnesium chlorides had no effect on the solubility of the sputum inhibitor. Barium caused precipitation which was accompanied by irreversible inactivation. However, it was possible to precipitate the inhibitor with little difficulty at low pH values in the presence of Ca^{++} at concentrations between 0·02 and 0·1 N. This reaction formed the basis of the method of purification of the inhibitor and the final procedure is described below.

Final method of purification of the sputum inhibitor. Semi-purified supernatant from the preliminary centrifugation was again centrifuged in the No. 30 rotor for 20 minutes at 35,000 *g*. The pH of the supernatant was adjusted by the addition of an equal volume of the pH 3·6 calcium acetate buffer. The resulting precipitate was removed by centrifugation at 3,000 *g* for 10 minutes in a refrigerated horizontal head centrifuge and dissolved in the pH 7·0 phosphate buffer. A slight precipitate of calcium phosphate was removed by centrifugation (3,000 *g* for 10 minutes). The pH was adjusted to 7·0 and the solution dialysed against normal saline. Prolonged dialysis was found to be necessary to obtain complete solution of the inhibitor.

The final product was a white opalescent solution. Solubility tests indicated that the inhibitor was very sparingly soluble below pH 5 (to the extent of 0·05 p.c. w/v). Both the solubility and the translucence increased with increasing pH. At pH 10 the inhibitor was soluble to the extent of 0·6 p.c. (w/v). It was found that the inhibitor titres of the solutions were proportional to the dry weights of inhibitor present. The purified inhibitor represented 25–35 p.c. of the dry weight of the original sputum.

Electrophoretic examination. Electrophoresis of the purified inhibitor in the phosphate buffer (pH 7) and the glycine buffer (pH 10) revealed a single peak with mobility $6{\cdot}9 \times 10^{-5}$ and $6{\cdot}0 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1} respectively (Figs. 1, 2). After treatment with RDE the mobilities are altered to $5{\cdot}0 \times 10^{-5}$ and $4{\cdot}5 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1} respectively (Figs. 1, 2). This apparently represents a change in electrophoretic mobility of all the material, that is, it is all substrate for RDE. Table 2 shows the inhibitor titres of a sample of the material used in the electrophoretic analysis before and after treatment with

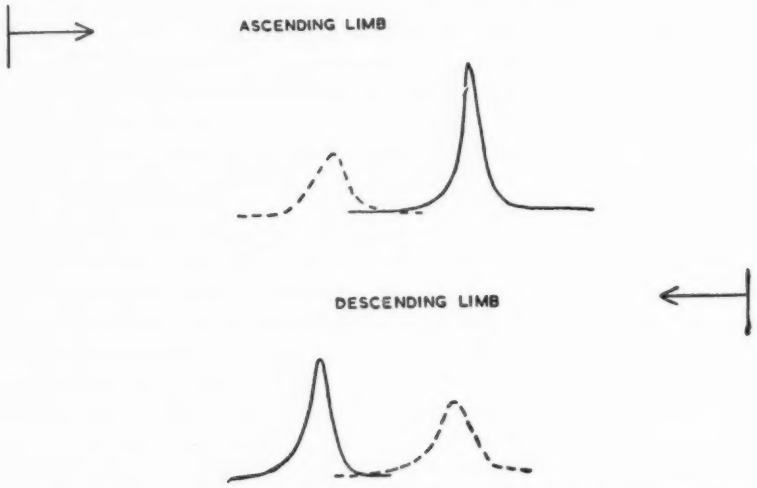

Fig. 1. Electrophoresis of the sputum inhibitor in phosphate buffer (pH = 7, ionic strength =0·1) for 1,480 seconds at 3·40 volt cm.$^{-1}$ before (————) and after (- - - - - - -) treatment with RDE. The vertical line and arrow refer to the initial position of the boundary and to the direction of migration.

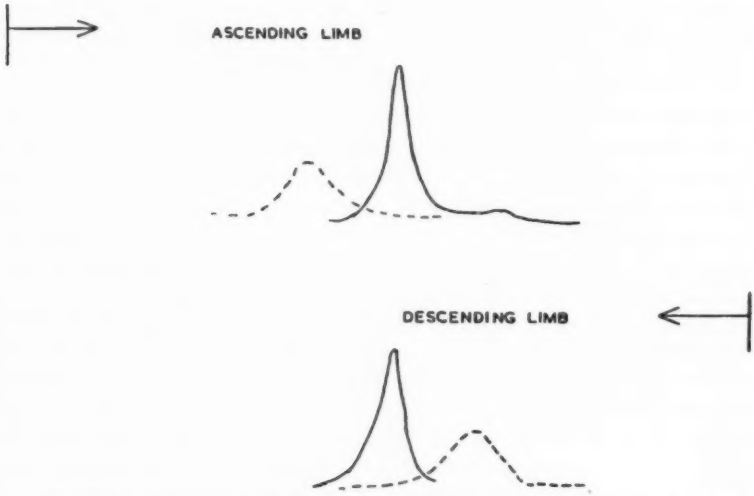

Fig. 2. Electrophoresis of the sputum inhibitor in glycine buffer (pH = 10, ionic strength = 0·1) for 2,020 seconds at 2·54 volt cm.$^{-1}$ before (————) and after (- - - - - - -) treatment with RDE.

RDE. As might be expected with a substance of pathological origin, several small batches (from 5 patients or less) had material of a lower electrophoretic mobility (concentration up to 40 p.c.) which was not a substrate for RDE. These batches were not used for the studies in this paper.

TABLE 2.

Haemagglutinin inhibitor titres of the sputum preparation used in electrophoresis in phosphate buffer.

	Viruses					
	MEL	LEE	WSE	T-MEL	H-LEE	H-WSE
Untreated sputum inhibitor	200	80	1,000	9,000	4,000	8,000
RDE-treated sputum inhibitor	10	10	10	10	10	10

The results in Table 2 also suggest that the inhibitions of haemagglutination by enzymically active and by "indicator" viruses are closely associated. Both are precipitated by calcium at pH 3·6 and both are removed by RDE. As only one component can be detected by electrophoresis and its mobility is changed as a whole by RDE, it seems that both properties are due to the same substance. The order of inhibitor titres against enzymically active viruses was PR8 = WSE > MEL > FMI > CAM > LEE.

Other inhibitors in sputum. Titrations of the precipitated, "purified" inhibitor and of its corresponding supernatant showed that a variable amount of inhibitor could still be detected in the supernatant (Table 3). It was of interest to find out if this inhibitor differed in any other respect from that which had been precipitated.

TABLE 3.

Haemagglutinin inhibitor titres of a batch of a precipitated inhibitor and its supernatant before and after RDE treatment.

		Inhibitor Titre (5 A.D.'s of virus)*						
		WSE	MEL	CAM	LEE	H-WSE	T-MEL	H-LEE
Supernatant	No RDE	200	10	15	10	1,500	1,500	1,000
	+ RDE	<10	<10	<10	<10	<10	<10	<10
Precipitate	No RDE	4,000	300	200	40	30,000	30,000	20,000
	+ RDE	<10	±10	±10	<10	<10	<10	<10

* All titrations in citrate saline.

A large volume of the supernatant from the final method of inhibitor purification was concentrated by pressure dialysis. This was submitted to methanol fractionation at 0° C. in the presence of pH 4·0 acetate buffer (0·08 M. acetic acid, 0·02 M. sodium acetate). Almost all of the inhibitor was found in the fraction which precipitated at 70 p.c. (v/v) methanol concentration. This

material was submitted to electrophoresis in the pH 7·0 phosphate buffer (Fig. 3). The pattern shows two main groups of components (mobilities about 5×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} and 3×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}). These are different from that of the purified sputum inhibitor and it is probable, therefore, that the inhibitor remaining in the supernatant is a different substance from that precipitated at pH 3·6 in the presence of calcium ions. On treatment with RDE the mobility of a considerable proportion of the supernatant fraction is reduced, indicating that it is largely inhibitor. Treatment with RDE also removes all the haemagglutinin inhibitor activity from the supernatant (Table 3).

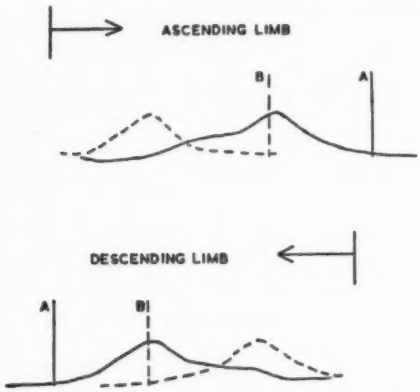

Fig. 3. Electrophoresis of the supernatant inhibitor in phosphate buffer (pH = 7, ionic strength = 0·1) for 860 seconds at 4·6 volt cm.$^{-1}$ before (————) and after (- - - - - - -) treatment with RDE. The vertical lines A and B represent the positions which would be occupied by boundaries corresponding to normal and RDE-treated sputum inhibitor respectively after electrophoresis under the same conditions.

The method of collecting the sputum would necessarily lead to the inclusion of saliva, as well as the bronchial secretions, in the samples. As an inhibitor is known to be present in saliva, it was of interest to learn if it differed from sputum inhibitor precipitated at pH 3·6 and if it was the same as that found in the supernatant.

Tests of saliva showed that no precipitate was formed in the presence of Ca^{++} at pH 3·6. Adjustment of saliva to pH 2 by addition of glacial acetic acid led to the precipitation of an inhibitor, but the same treatment did not precipitate inhibitor from the super natant left after the preparation of purified sputum inhibitor.

It was concluded, therefore, that the inhibitors in the sputum preparations were not the same as that in saliva.

PART II.

Neutralizing properties of sputum preparations for the infectivity of influenza virus in the allantoic cavity.

Sputum from a number of individual patients was processed to the "semi-purified supernatant" stage (a preparation containing both sputum inhibitors) and titrated for ability to neutralize infection by influenza virus (WSE) in the allantoic cavity of the chick embryo. The WSE strain of virus was chosen

because its enzymically active form was inhibited to the greatest extent in the *in vitro* haemagglutination-inhibition tests of sputum and it was thought that if the effect depended on the mucins this ought to give the maximum neutralizing effect. The inhibition titres given *in vitro* by sputum preparations and PR8, the influenza virus used by Rose (1950), were of a similar order to those given by WSE. Sputum samples from seven patients were examined. Of these, two showed no neutralization, two showed neutralization of virus up to 1,000 ID_{50}'s and three showed neutralization values greater than this. One of these latter specimens (No. 4) was from a patient with chronic bronchitis and carcinoma of the stomach and was used for further investigation of the neutralizing factor.

Behaviour of neutralizing factor with "O" phase strain of influenza virus. The pooled amniotic fluids of two eggs of a group inoculated with a gargling from a patient with influenza were used as a source of "O" phase virus. Their human/fowl cell haemagglutination titres were $320/< 10$ at 4° C. Certain ten-fold dilutions of the virus were mixed with equal quantities of the sputum sample and after incubation at 37° C. for 30 minutes, inoculated in 0·1 ml. amounts into groups of 13-day chick embryos by the amniotic route. Sputum sample 4, which neutralized $10^{5 \cdot 25}$ ID_{50}'s of WSE in the allantoic cavity, showed a neutralization value of $10^{0 \cdot 75}$ in the amniotic sac with the "O" phase virus. The sample inhibited haemagglutination by active and "indicator" states of "O" phase virus to titres of 80 and 10,240 respectively.

Other experiments showed the neutralizing factor was equally effective against virus (WSE or CAM) in the allantoic cavity regardless of whether the sputum-virus mixtures were held at 37° C. or 0° C. before they were inoculated into eggs.

The possibility that components of saliva mixed with the sputum were responsible for the neutralizing effect was unlikely as neutralization was not observed with saliva alone.

Part III.

Relationship between neutralizing and inhibitory activities.

Preparations of the purified inhibitor were studied for neutralizing activity. In most cases no neutralization occurred, but some batches showed neutralizing values up to 10^4 ID_{50}'s of virus (CAM). The inhibitor titres of these precipitated preparations were of the same order as those with no neutralizing ability. Preparations with neutralizing activity did not lose it when treated with purified RDE, which removed all their inhibitor activity. It was probable, therefore, that the neutralizing ability found in some batches was due to contaminating substances precipitated with the inhibitor and not to the inhibitor as such. The following experiments were carried out in order to determine further the relationship between neutralizing and inhibitory activities.

Action of RDE on the neutralizing activity of sputum. If the neutralizing activity of whole sputum were dependent on the adsorption of virus to the receptors of either of the inhibitors present, then this ability should be removed when the receptors are destroyed, e.g. by RDE.

3·5 ml. of semi-purified supernatant from sputum sample No. 4 was mixed with 0·5 ml. of the calcium acetate buffered saline containing 100 units of penicillin and 0·1 mg. of streptomycin per ml. The mixture was divided into two 2 ml. lots. To one was added 400 units of purified RDE (vol. 0·05 ml.). To the other was added 0·05 ml. of saline. Both were incubated for 3 hours at 37° C. After incubation RDE-treated and control samples were titrated in citrate saline to determine their haemagglutinin inhibitor titres against five haemagglutinating doses of enzymically active and "indicator" WSE. Their neutralizing capacity *in ovo* was tested using the WSE strain of influenza virus. In this test the RDE-treated sample was divided into two portions. To one portion was added anti-RDE serum (previously inactivated at 60° C. for 30 minutes) in sufficient quantity (0·05 ml.) to neutralize the 200 units of RDE per ml. which had been put in originally. This was done to exclude the possibility of any modification of the titration by the action of residual RDE on the cells of the allantoic cavity when the mixtures were inoculated into eggs. The other portion was tested without the anti-RDE serum. Table 4 shows the results obtained.

TABLE 4.

Neutralizing capacity of semi-purified supernatant from sputum sample 4 before and after treatment with purified RDE.

	In vitro haemagglutinin inhibition titre to		50 per cent. infecting doses of virus (WSE) neutralized
	WSE	H-WSE	
Sputum sample 4 untreated	1,920	10,240	$10^{5 \cdot 25}$
Sputum sample 4 + RDE	5	±10	$10^{4 \cdot 5}$
Sputum sample 4 + RDE + anti-RDE serum	—	—	$10^{4 \cdot 5}$

It can be seen that although almost all the *in vitro* haemagglutination inhibitor activity was removed by treatment with RDE, the *in ovo* neutralization value has only declined by 0·75 of a $\log_{10}$ unit.

This failure of purified RDE to remove the neutralizing ability of sputum, or sputum fractions, was found consistently with other samples of sputum and other test viruses.

Effect of trypsin on haemagglutinin-inhibitor and virus neutralizing factor. Semi-purified supernatant from sputum sample 4 was mixed with the pH 8·0 calcium borate buffer and crystalline trypsin and incubated in a 37° C. water bath. A control preparation in the same buffer, but without trypsin, was incubated under the same conditions. At the end of twenty-four hours' incubation

a preparation of the purified trypsin inhibitor was added to both trypsin-treated and control samples so that the trypsin would not interfere with subsequent virus titrations. The samples were then titrated for haemagglutinin inhibitor and for neutralizing ability using WSE. Table 5 shows the results obtained.

Treatment with trypsin decreased the neutralizing power of the sputum by at least 4·3 $\log_{10}$ units (i.e. virtually removed all activity) but the *in vitro* haemagglutination inhibitor titre was only reduced to about a third of its original value.

TABLE 5.

Effect of crystalline trypsin on the haemagglutinin inhibitor and neutralizing activity of sputum sample 4.

	In vitro haemagglutinin inhibition titre to		50 per cent. infective doses of virus (WSE) neutralized
	WSE	H-LEE	
Sputum sample 4 untreated (+ trypsin inhibitor*)	1,280	10,240	$10^{3\cdot7}$
Sputum sample 4 + trypsin (+ trypsin inhibitor*)	320	3,840	$\leqq 10^{1\cdot4}$

* Added at the end of the 24-hour incubation period.

A similar result with trypsin treatment was obtained using the influenza virus strain CAM and sputum with neutralizing properties obtained from a different patient (W).

Effect of heat on the neutralizing factor. Samples of sputum W (from one patient) were placed in ampoules and submerged in a water-bath at 56° C., 80° C. and 100° C. for 30 minutes. They were then titrated using the virus CAM. Table 6 shows neutralization values obtained.

TABLE 6.

Effect of heat on neutralizing factor of sputum.

Sample W	50 per cent. infective doses of virus (CAM) neutralized
Unheated	$10^{4\cdot87}$
56° C. for 30 minutes	$10^{3\cdot8}$
80° C. for 30 minutes	$10^{3\cdot8}$
100° C. for 30 minutes	$<10^{1\cdot0}$

The neutralizing activity was removed by heating at 100° C. for 30 minutes. In general, the haemagglutinin inhibitor titre against "indicator" viruses was not altered by heating at 100° C. for 30 minutes but the titres against enzymically active viruses fell to about a third of their original values.

Conclusion. The results of the treatments with RDE, crystalline trypsin and heat show that the properties of *in vitro* haemagglutination inhibition and *in ovo* neutralization can be diminished or abolished independently of each other.

DISCUSSION.

The investigations reported in this paper indicate that sputum collected from patients with chronic bronchitis contains two inhibitors of influenza virus haemagglutination and, inconstantly, a factor which will neutralize infection by some influenza viruses in the allantoic sac of the chick embryo. The inhibitors are active against both the enzymically intact and "indicator" states of the virus and these properties are completely removed by treatment with purified RDE but only modified by heat or trypsin. The inhibitors have different electrophoretic mobilities and one inhibitor, constituting 25–35 p.c. of the dry weight of the sputum, can be precipitated at pH 3·6 in the presence of calcium ions.

The neutralizing effect on infection *in ovo* which can be observed with specimens of sputum from some patients is apparently not due to the specific receptor groupings of the haemagglutinin inhibitors. Thus, the neutralizing factor is destroyed by heat and trypsin, is resistant to the action of purified RDE and the intensity of the effect is not increased by measures such as precipitation at low pH or ultracentrifugation which concentrate the haemagglutinin inhibitors. The factor does not appear to be active against "O" phase virus in the amniotic cavity but this route may be less satisfactory for neutralization tests.

Our findings concerning the inconstant, labile nature and heat sensitivity of the neutralizing factor are in agreement with those of Rose (1950) although, unlike him, we attribute the capacity "to inhibit virus haemagglutination" (i.e. presumably haemagglutination by enzymically active virus) to the inhibitors rather than to the neutralizing factor.

The identity of the factor which neutralizes the infectivity of virus is undecided. Rose (1950) states that it is not specific antibody but this possibility does not seem to have been finally excluded. Somewhat similar findings have previously been reported with human nasal secretions. Burnet, Lush and Jackson (1939) found that nasal secretions would inactivate the infectivity of influenza virus, and also that of herpes and louping-ill. The agent (V.I.A.) responsible was active at 26° C. but not at 0° C., and destroyed by heating (100° C. for 10 minutes). A series of investigations by Francis and his colleagues (see Francis, 1941) showed that the agent in nasal secretions varied in quantity from person to person, being more frequently present in those with serum "antibody" to the test virus; that it was inactivated at 70 to 76° C., destroyed by trypsin and precipitated by half saturation of the solutions with ammonium sulphate. In addition, the activity of secretions was markedly increased in persons who had been vaccinated with influenza virus. In this connection it is of interest that Mulder, Brans and Hers (1952) have recently shown that a haemagglutinin inhibitor for enzymically active virus, not destroyed by crude

RDE and, therefore, probably antibody, increases in amount in the nasal secretions of persons following vaccination with influenza virus. So with the exception of the discrepant finding of Burnet *et al.* (1939), that V.I.A. is inactive at 0° C.—which does not obtain with sputum neutralizing factor—these findings with nasal secretions, like those obtained with sputum, are compatible with the view that the neutralization observed is, in fact, due to specific antibody. Another, more remote possibility, which is being investigated, is that the neutralization is due to a natural antibody or "non-specific inhibitor" of the non-mucoprotein type described by Burnet and McCrea (1946) and Chu (1951) in animal sera.

The neutralization effect observed in these experiments with whole sputum preparations was small; values were rarely more than 10^4 to 10^6 ID_{50}'s of virus neutralized in the constant sputum-varying virus test. This test would seem, from experiments carried out with a convalescent serum from a patient suffering from influenza, to be of a sensitivity which still enables "antibodies" to be detected when other methods such as haemagglutinin-inhibition, or complement fixation with virus-infected allantoic fluid, give negative results. Consequently, it is possible that sputum preparations could contain enough antibody to give an *in ovo* neutralizing effect of the small degree observed without convincing levels of RDE-resistant haemagglutinin inhibitor or complement fixing antibodies being detectable.

The absence of *in ovo* neutralizing effect due specifically to the sputum inhibitors is in line with the findings obtained with mucoprotein inhibitors in general. If certain inhibitors are modified by treatment with periodate, so that virus is adsorbed but cannot elute from their receptors, they then develop an *in ovo* neutralizing activity (Burnet, 1948, 1949). This suggests that the enzyme is of value to the virus in releasing it from inhibitors and freeing it to combine with cell receptors, so enabling infection to take place—at least, that is, in the artificial circumstances of the *in ovo* neutralization test.

The effect of the inhibitors on small amounts of virus landing on the mucous film of the respiratory tract under natural conditions of infection may, of course, be different. Fazekas (1952) has shown that the receptors of nasal mucins are modified or destroyed during active influenzal infection in the human. But this effect is (relatively) late in the process when infection of many of the respiratory cells has already taken place and large amounts of virus are free in the respiratory tract.

In further investigations of the possible modifying effects of bronchial mucus on the initial contact of virus and susceptible cell, a distinction has to be made between the effect due to inhibitors and to the neutralizing factor in sputum. The ability to prepare pure preparations of sputum inhibitor may be of value in such an analysis.

SUMMARY.

The effect of preparations of human sputum on the haemagglutinin and infectivity of influenza virus has been investigated.

Sputum was found to contain two inhibitors of virus haemagglutination and a factor which neutralized infection by virus in the chick embryo.

The inhibitors had different electrophoretic mobilities and one, constituting 25–35 p.c. of the dry weight of the original sputum, was extracted in a purified form by precipitation at low pH in the presence of calcium ions.

The inhibitors were shown to be active against both enzymically intact and "indicator" virus *in vitro* but did not neutralize infection by virus in the egg.

Some properties of the neutralizing factor are described and its relation to specific influenza antibody is discussed.

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RECOMBINATION STUDIES WITH TWO INFLUENZA VIRUS B STRAINS

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A large amount of work on genetic interaction between influenza virus A strains has now been carried out in this laboratory. The first experiments involved the intracerebral inoculation in mice of mixtures of the neurotropic influenza virus NWS with a normal strain (MEL, SW or OC.1.) of different serological sub-type. In each series it was possible to obtain virus which after reisolation by passage in eggs at limiting infective dilution had the capacity to produce encephalitis in mice combined with the serological character of the non-neurotropic component of the mixed infection (Burnet and Lind, 1951 a, b).

The obvious interpretation was that a process of genetic interaction between two influenza A viruses could occur, presumably when simultaneous infection of the same cell was effected. In the mouse brain system any new types of virus would be subjected to strenuous competition for survival and only forms with some special virtue in that respect would be isolated. At that time it seemed necessary if one were to isolate a recombinant type that an environment or sequence of environments should be provided in which neither parent strain could flourish but which would allow a particular type of recombinant to multiply.

Limited experiments had failed to show any interaction when a B strain LEE was used in similar experiments with NWS. An attempt was therefore made to devise an analogous experiment by which genetic interaction between two B strains could be recognized. No neurotropic B strain is available so use had to be made of the differing pathogenicity for the mouse lung of adapted and unadapted strains. The two B strains which we maintain as part of our stock series of "gradient" viruses, LEE and MIL, differ in serological sub-type, resistance of the haemagglutinin to heat, position in the receptor gradient and pathogenicity for the mouse lung. The primary experiments were designed to determine whether from mixed inocula with a great excess of MIL adminis-

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tered intranasally, mouse pathogenic strains with MIL antigenicity could be isolated. This type of experiment has been almost regularly successful in producing such "lung pathogenic MIL" strains which are not obtainable from lungs inoculated under similar conditions with MIL alone.

While this work was in progress an approach to recombination studies involving primary interaction in the cells of the allantoic membrane was being developed (Burnet and Fraser; Burnet and Lind, 1952). The results obtained, especially the demonstration by Burnet and Lind of reciprocal recombinants from mixed infections by the strains WSE and MEL, led to further elaboration of the present work. A re-examination of one of the primary lung emulsions showed that a proportion of "non pathogenic LEE" strains could be isolated. Passage of "pathogenic MIL" through mice gave rise to one strain of highly unstable character from which were obtained a wide variety of descendant clones illustrating almost every combination possible of LEE and MIL qualities. All three types of result point strongly toward the conclusion that genetic interaction between two influenza B strains can occur with the production of several types of recombinant.

Materials and Methods.

Virus strains. Two strains of influenza B virus were used: LEE—classical strain, and MIL—isolated during an epidemic in Melbourne in 1945. Virus was grown in the allantoic cavity of eleven-day embryonated eggs, the allantoic fluid was harvested after two days' incubation. Fluids used were obtained from eggs inoculated with a high dilution of glycerolated seed virus so that these fluids represented pure clones of virus (Burnet, 1951). In the following experiments virus was used either as freshly harvested allantoic fluid, or after this fluid had been stored for a time in the dry ice cabinet.

Haemagglutinin titrations. Serial doubling dilutions of virus were made in 0·25 ml. volumes of saline, and an equal volume of 1 p.c. fowl cells added. The tests were allowed to settle at room temperature, and the usual + degree of partial agglutination taken as the end point. The dilution at the end point contains one agglutinating dose of virus.

Inoculation of mice. Mice used were between four and five weeks of age. Inoculation was by the intranasal instillation of 0·05 ml. of virus given while the mice were under light chloroform-ether anaesthesia. For pathogenicity tests mice still alive seven days after inoculation were killed and examined for lung lesions. The method of grading lesions is given under Table 2. For virus isolation lungs were removed from mice four days after inoculation. These lungs were ground with sterile silica, suspended in 0·5 ml. of 10 p.c. horse serum saline per pair of lungs, and lightly centrifuged. Penicillin was added, and dilutions of the supernatants were used for egg inoculations.

Characterization of virus fluids.

The virus strains obtained were examined for each of the qualities in which a standard MIL fluid differs from a LEE fluid.

(A) Serological character as tested against immune ferret sera. The values given in Table 1 represent the dilution of immune ferret serum just neutralizing the haemagglutinating activity of 5 ADs of virus.

(B) Heat resistance of haemagglutinin. LEE fluids heated to 62°C. for 30 minutes show a fall of HA titre only to about one half the original. MIL fluids are reduced to 5 p.c. or less of the original titre.

(C) Position in the receptor gradient. The method used is that described by Isaacs and Edney (1950) in which human cells are treated for 30 minutes at 37° C. with graded dilutions of a stock preparation of crude RDE and subsequently washed with citrate saline. The end point given is the dilution of RDE which gives cells that are agglutinated to + level of partial agglutination by 5 ADs of the virus fluid being studied. MIL is much lower in the gradient than any other strain in our possession and the value given it is correspondingly small.

(D) Pathogenicity for mice intranasally. Except in the first experiments, all tests were made with virus diluted to 1 AD, i.e., if a fluid gave an HA titre of 100 by the standard method, 0·05 ml. of a 1:100 dilution was inoculated. Four mice (4–5 weeks) were used for each fluid and the lesions examined at 7 days.

TABLE 1.

Characters of representative LEE and MIL strains of influenza B virus.

Strain	Haemagglutinin		AHA titre of		Gradient on RDE cells	Lesions in mice inoc. 1 AD
	Original	H 62°	Anti-LEE F 372	Anti-MIL F 371		
LEE	560	240	320	120	48	4,4,4,3 (3·75)
MIL	140	<2	60	270	2	0,0,0,0 (0·0)

Table 1 summarizes the findings when these tests are applied to the standard strains and provides a basis for comparison with the strains subsequently obtained.

EXPERIMENTAL RESULTS.

The isolation of mouse-pathogenic MIL strains from double infections.

Two experiments only will be described in any detail. Experiment 1 is concerned in establishing that growth of MIL in the mouse lung does not give rise to any detectable virus with the character of the "recombinant" obtained in the second type of experiment in which mixtures with LEE were used. A typical example of such an experiment is described as Experiment 2.

Experiment 1. Six mice were given MIL allantoic fluid diluted one in ten, and the lungs removed from two mice four days later, and treated as under "Materials and Methods." None of the remaining four mice killed seven days after inoculation showed any lung consolidation. Eggs were inoculated with dilutions of the supernatant from the mouse lung suspension. Only small amounts of virus were present, the ID_{50} being approximately 10^{-2}. Two positive fluids from dilutions 10^{-2} and 10^{-3} were tested. The original lung emulsion which had been stored in a dry ice refrigerator in the interim was re-tested at dilutions of 10^{-1} and $10^{-2\cdot5}$ using larger numbers of eggs. Fourteen out of twenty embryos were infected at 10^{-1}, none at $10^{-2\cdot5}$, and 12 of the fluids were

tested for the differentiating characters described under "Materials and Methods." The results are summarized in Table 2. All the fluids tested showed the usual properties of MIL.

TABLE 2.

Character of virus fluids isolated from mice inoculated with MIL alone.

Mouse lung dilution	Proportion of + fluids	No. tested	Serology	RDE cells	Mouse pathogenicity
10^{-1}	4/4 14/20	12	MIL	3–8	0·15, 0·12, 0·12, 0, 0, 0, 0, 0 0, 0, 0, 0
10^{-2}	1/4	1	MIL	3	0
10^{-3}	1/3	1	MIL	6	0

Mouse lung lesions were graded:

4 = complete consolidation with death of mouse on 6th or 7th day. (Mice dying on 4th or 5th day were given a value 5.)
3 = more than half lung consolidated.
2 = approximately half the visible area of the lung surface consolidated.
1 = about 1/6 to 1/4. 0·5, 0·2 and tr for smaller areas of damage.

The mean value for 4 mice is given in the Tables.

In all experiments of the type of Experiment 2 below, at least one fluid isolated from mice inoculated with MIL alone was tested for mouse pathogenicity. All were non-pathogenic with no lesions above the "tr" level with 1 AD of virus inoculated.

In one experiment, five fluids derived from the titration of lungs from mice inoculated with MIL alone were tested. One of these, 77163, was exceptional in two respects. It gave relatively marked lung lesions averaging 2·6, and was at the opposite end of the receptor gradient. On further passage in chick embryos, this strain retained its position in the gradient near MEL, but showed lower mouse pathogenicity, three fluids giving mean values of 0·3, 0·6 and 0·3 in mouse lung lesions. The strain was checked carefully to ensure that it was serologically MIL and not a contaminating virus. In none of the experiments in which a mixture of LEE and MIL was employed was a fluid directly isolated which had this high receptor gradient position combined with the serological character of MIL; however, passage of a pathogenic MIL strain, shown in the next section, has yielded one fluid with this characteristic. This strain is considered to be a mutant, and is being retained for further study.

Experiment 2. Three groups of six mice were inoculated intranasally, A with MIL diluted 10^{-1}, B with a mixture containing final dilutions of MIL $10^{-1·3}$ and LEE $10^{-4·8}$, and C with LEE diluted $10^{-4·5}$. Four mice examined for lung lesions a week later showed mean scores as follows: A (MIL) 0·75, B (MIL + LEE) 2·3, C (LEE) 0·75. Lungs from two mice in each of groups A and B were removed at 4 days and suspensions titrated in chick embryos. In order to allow preferential growth of MIL, one set of titrations of B (MIL + LEE) was done using as diluting fluid a 1:10 dilution of LEE antiserum. The result of this preliminary titration is shown in Table 3.

TABLE 3.

Virus content of mouse lung emulsions.

Lung suspension	Diluent	Dilutions 10−1	10−2	10−3	10−4	10−5	10−6
A (MIL)	NHSS		1/4a	0/4	0/4	0/4	
B (MIL + LEE)	NHSS	4/4	4/4	3/3b	2/3c	0/3	
B (MIL + LEE)	Anti-LEE	4/4	3/4d	3/4e	0/4		

Serological type a MIL: b 2 LEE: c 2 LEE: d 3 MIL: e 3 MIL.

In order to obtain a larger number of fluids for characterization, suspension B after being held in the dry ice cabinet was retitrated in 1:10 LEE antiserum at dilutions 10^{-2}, $10^{-2\cdot5}$ and 10^{-3}. With 15 eggs at each dilution, 6, 3 and 2 positive fluids were obtained respectively. All were serologically MIL. The results of testing fluids from both parts of this experiment are shown in Table 4.

TABLE 4.

Character of virus fluids isolated from mice inoculated with a mixture of LEE and MIL.

Mouse lung dilution	Proportion of positive fluids	Fluid numbers	Haemagglutinin Orig.	Haemagglutinin H 62° C.	AHA titre Anti-LEE F372	AHA titre Anti-MIL F371	Gradient on RDE cells	Mouse pathogenicity
		79412	270	<2	60	270	3	1·5, 1, tr, 2 (1·1)
		79413	20	<2	60	270	5	tr, tr,2,tr(0·5)
10−2	3/4 6/15	79415	25	15	—	—	2·7	4,2,2·5,2·5 (2·75)
		79420	80	<2	64	320	3	4,1,3 (2·7)
		79426	10	<2	—	240	2	0,0,tr,0 (0)
		79428	200	15	60	270	3	0·5,3·9,3·5,4(3)
		79435	50	<2	60	270	3	4, 4, 4, 3 (3·75)
10−2·5	3/15	79440	20	2	40	270	6	4, 3, 3, 4 (3·5)
10−3	3/4 2/15	79447	140	13	70	240	2·7	3·5, 2, 3·5, 4 (3·25)
		76532	120	—	110	280	4	4, 2, 3, 2 (2·75)
		76533	960	—	120	270	4	0·5, 0·5, tr, 1(0·5)

The tests for mouse pathogenicity show that three levels of activity can be recognized. Three strains, 79413, 79426 and 76533, are indistinguishable from standard MIL. Seven have high pathogenicity averaging grade 3 lesions per AD, while one, 79412, is of intermediate quality.

Fluid 76532 was inoculated into eggs, and one fluid, 76916, the only fluid positive out of four eggs at 10^{-7} dilution, was inoculated again into eggs. Three out of four positive fluids were obtained from this passage at 10^{-5} dilution, and these were tested for the standard properties, results shown in Table 5. It

can be seen that the strain has retained its properties of mouse pathogenicity through two egg passages at limiting infective dilutions though the extent of the lesions produced is slightly less than was found with the parent fluid.

TABLE 5.

Character of virus fluids from the second egg passage of fluid 76532.

Fluid No.	AHA titre		Gradient on RDE cells	Mouse pathogenicity
	LEE antiserum F370	MIL antiserum F371		
77109	80	240	11	4,1,1,1 (1·75)
77110	60	240	12	1,3·5, 2,2 (2·1)
77111	60	210	12	3,2·5,2,0·5 (2·0)

Three other experiments similar to the one just described were carried out. In all of these, strains pathogenic for mice, but otherwise having MIL characteristics, were isolated. In the first experiment two fluids of this type were obtained while one other fluid tested gave LEE characters. It may be noted that all three fluids were obtained by direct titration of the mouse lung suspension in embryos without the use of LEE antiserum. The two MIL strains were inoculated intranasally into mice, re-isolated, and passaged twice in eggs at limiting dilutions. One fluid of the second egg passage of each strain was tested for the standard properties. Both strains had the serological characters of MIL; in mice the following lesions were obtained—with one fluid tr, 4,tr, tr and with the other 3,3,0·5, 4.

In the second experiment one of three fluids tested, all of which showed MIL characteristics, was pathogenic for mice. This fluid was carried through two egg passages at limit dilutions, and three fluids tested from the last passage showed good mouse pathogenicity, lesions averaging 3·0, 2·9 and 2·3.

In the third experiment a mixture of fluids was isolated, some LEE and some MIL, one fluid apparently MIL, but showing mouse pathogenicity was passaged in eggs, and the fluids positive at the limiting infective dilution tested—of three fluids one was LEE and the other two having MIL serology were pathogenic for mice.

Mouse passage of pathogenic MIL strain.

Two of the fluids showing the new combination of characters (Nos. 79435 and 79440 of Table 4) were carried through four intranasal passages in mice to establish whether the mouse pathogenicity remained the same. When the series was completed lung emulsions of the fourth passage were titrated in eggs and the LD fluids characterized. All but one were of the type expected. The exception, however, had a completely different set of characters and on further

titration gave four distinct combinations. As this provides evidence from a wholly unexpected quarter of very literal recombination of characters we have felt that it should be put on record.

Fluids 79435 and 79440 were first titrated in eggs and LD fluids 82736 and 82545 respectively chosen for passage in mice. For the first intranasal inoculation 1 AD was used; subsequent passages were made using suspensions of lungs removed at 2 or 3 days. Other mice of each passage were killed at 7 days and the lesions recorded. The 4th set of lung emulsions were titrated in the allantoic cavity and 3 LD fluids from each characterized. Those derived eventually from 79440 were all similar to the parent except that the lung lesions were significantly less severe, being graded 1·6, 1·25 and 1·25. The fluids derived through mouse passage from 79435 are shown in Table 6.

TABLE 6.

Character of LD fluids obtained after several passages of fluid 79435 in mice.

Origin	Fluid No.	HA titre		AHA titre		RDE	Mouse
		Orig.	H 62° C.	LEE F370	MIL F371	Gradient	pathogenicity
Stock	LEE	600	400	640	80	>64	2·75
„	MIL	240	<2	70	240	6	0·1
79435	83372	450	<2	33	200	6·5	1·2
„	73	240	2	50	240	4	2·25
„	75	960	340	60	480	>64	3·0

Fluid 83375 has all the characters of LEE except that it is still quite definitely MIL serologically. This fluid was titrated and six fluids obtained at LD characterized. In addition to stock LEE and MIL fluids, two LD fluids from the atypical fluid 77163 described previously were included in this series of tests.

TABLE 7.

Characteristics of MIL variants.

Origin	Fluid No.	HA titre		AHA titre		RDE gradient	Lung lesions in mice
		Unheated	Heated 62° C.	LEE F370	MIL F377		
Stock	LEE	640	480	960	120	48	3·9
„	MIL	480	<5	60	240	3	0·0
77163	86003	480	80	60	450	48	0·1
„	006	450	80	60	450	48	0·6
83375	86007	400	320	50	200	8	3·4
„	08	960	7	60	300	50	3·75
„	09	120	160	60	300	12	0·0
„	10	1,000	120	60	400	50	3·25
„	11	850	240	60	400	50	3·1
„	12	1,000	480	60	300	50	3·0

These results can be grasped more easily if we label the four differentiating characters as follows: Aa, serological type; Bb, resistance of HA to 62° C.; Cc, position in receptor gradient; Dd, pathogenicity for mice. LEE will have the formula ABCD, MIL abcd, and 77163, a(b)Cd.

Then the sequence with which we have been concerned is

(LEE) ABCD + abcd (MIL)
↓
abcD (79435)
LD egg passage ↓
abcD
Four mouse passages ↓
abcD (2 fluids) aBCD (83375)
LD egg passage ↓
aBcD abCD aBcd a(B)CD
86007 86008 86009 86010, 11 and 12

A second extensive retitration of fluid 83375 gave a somewhat different pattern. Of the fluids completely tested, one was abcD. two abCD, two aBCD, while five showed intermediate heat stability ab/BCD.

Further titration of the four types represented in Table 7 gave the following LD fluids, the characters being expressed in the same convention. Where a symbol is bracketed, e.g. (b), the character in question was intermediate but nearer b than B, (C) intermediate but nearer C than c.

86007, aBcD, gave 1 aBCD and 4 abcD
86008, abCD, gave 4 a(B)CD and 1 aBCD
86009, aBcD, gave 1 a(B)cd, 1 abcd and 2 a(B)(c)d
86010, a(B)CD, gave 3 aBCD and 3 a(B)CD

It will be seen that while in each case the antigenic character and pathogenicity for mice has remained constant, the heat stability and position in the receptor gradient have been variable. If we include 77163 and its descendants (abCd) there are very few of the possible combinations with MIL antigenic character that have not been obtained.

Reciprocal type of recombinant from doubly infected mouse lungs.

In view of Burnet and Lind's (1952) results, it was thought advisable to make a restudy of some of the mouse lung emulsions to see whether in addition to lung pathogenic MIL strains, non-pathogenic LEE might be found. Mouse lungs 136–7, used in the last experiment recorded in the first section, had been stored as a saline emulsion in the dry ice refrigerator for approximately a year. The virus content of the emulsion had not fallen and the following fluids were tested: 10^{-3}, 5/5, 10^{-4}, 4/6. In terms of the symbols used in the preceding section the types of fluid obtained were:

1 A(B)CD, 3 ABC and 1 A(B)C (Dd not tested), at 10^{-3}.
ABCD, ABCd, A(B)C(d), abc(D), at 10^{-4}.

TABLE 8.

Character of LD fluids from retitration of mouse lung.

Mouse lung dilution	Proportion of positive fluids	Fluid numbers	Haemagglutinin Orig.	Haemagglutinin H 62° C.	AHA titres Anti-LEE F372	AHA titres Anti-MIL F377	Gradient on RDE cells	Mouse pathogenicity
	Stock	LEE	530	480	400	75	>64	4,4,3·5,3(3·6)
	Stock	MIL B	80	<4	<50	100	6	0,0,0,0, (0·0)
		92754	560	480	330	83	>64	tr,tr,tr,0(tr)
		92755	960	240	300	67	>64	0·5,tr,1,2·5(1·0)
10^{-4}	4/6	92756	480	<20	<50	150	12	1·5,0·5,tr,1·5(0·9)
		92757	480	420	530	75	>64	3·5,3,4,3·5(3·25)

It will be seen that in the four LD fluids one fluid (92754) has the *in vitro* qualities of LEE but lacks pathogenicity for the lung. In addition there is a MIL strain with increased though not full pathogenicity. This fluid, 92754, was passaged again through eggs, and the LD fluids characterized—$\frac{3}{4}$ eggs were positive at 10^{-7} dilution. The three fluids were of the following types:

2 ABCd and 1 A(B)Cd.

DISCUSSION.

These results show that when double infections of two influenza B virus strains are produced in the mouse lung, new types can be isolated in pure clones which give every indication of being recombinants. It is hard to see any other explanation for the simultaneous isolation from infected mouse lung of the two original types and two reciprocal recombinants. Expressed in the convention we have used in this work the results to be interpreted are:

$$\frac{\text{ABCD} \times \text{abcd}}{\text{ABCD} \quad \text{ABCd} \quad \text{abcD} \quad \text{abcd}}$$

While the appearance of abcD (pathogenic MIL) could be regarded as the selective survival of a rare spontaneous mutant, no such explanation is available for the non-pathogenic LEE (ABCd). As in other such situations the only alternative explanation must be that in some way there is a mutual interference between the normal forms which allows rare variants of both to appear. Quantitative considerations eliminate this alternative where, as in Burnet and Lind's (1952) experiments, the first cycle yield only is considered; but where several cycles are possible, as in the present experiments, it remains a theoretical if very unlikely alternative.

The appearance in one series of passages of the change abcD–aBCD followed by a marked instability in characters Bb and Cc in the descendants, would, if

taken at its face value, demand that the abcD strain was in fact a diploid form (abcD–aBCD) which after one LD passage in eggs and four bulk passages in mice gave rise to segregants of unstable character. In view of Hershey and Chase's (1951) finding that heterozygotes amongst bacterial virus recombinants never multiply as such, this explanation seems unlikely. Until recombination between influenza viruses and the associated phenomena which we have ascribed to "heterozygous virus" are more clearly understood, we feel it is advisable to put the facts on record without attempting any interpretation.

SUMMARY.

Intranasal infection of mice with appropriately balanced mixtures of the two influenza virus B strains LEE and MIL, has allowed the isolation of two reciprocally related new forms which are interpreted as recombinants.

Passage of one of these recombinant types gave rise to a series of genetically unstable variants amongst which could be found nearly all the possible combinations of the marker characters other than serology which distinguish LEE from MIL.

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A SURVEY OF THE OCCURRENCE OF CARDIO-ACTIVE CONSTITUENTS IN PLANTS GROWING WILD IN AUSTRALIA

I. FAMILIES *APOCYNACEAE* AND *ASCLEPIADACEAE*

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The cardiac glycosides represent one of the few remaining groups of drugs derived solely from plant sources and for this reason are the subject of considerable research at the present time.

We have attempted to survey the occurrence of these drugs in Australian plants as part of the Australian Phytochemical Survey of the Commonwealth Scientific and Industrial Research Organization.

So far the families *Apocynaceae* and *Asclepiadaceae* have been almost completely examined from material collected in Queensland.

In order to obtain a quick evaluation of these plants the anaesthetized guinea pig has been used and extracts of the plants administered by continuous intravenous injection. This is essentially the official method of the British Pharmacopoeia (1948) for the assay of digitalis, and it provides a means of detection of almost any substance which has a direct action on the cardio-vascular system.

A cardiac glycoside injected in this way quickly slows the heart and makes the individual contractions more forceful. With more prolonged administration cardiac arrhythmias are observed with dropped beats and coupling of the auricular and ventricular contractions when the heart is very slow. This later gives place to ventricular fibrillation and finally cardiac arrest.

Some substances which are purely toxic to the heart cause an increase in rate together with increasingly feeble contractions. This is followed by progressive poisoning of the heart until it fails in diastole. With toxic effects of this kind no slowing or increase in ventricular contractile force is observed at any time during the experiment and it is reasonable to assume the absence of cardiac glycosides in such circumstances.

METHOD.

The plant specimens, unless otherwise stated, were received in air-dried condition, the drying having been done at 54–60° C. for varying lengths of time.

The plants were ground to a coarse powder and 25 gm. of the product were macerated with 70 p.c. alcohol for 48 hours. The alcoholic extract was then separated by filtration and concentrated *in vacuo* until the alcohol was almost completely removed. The concentrated extract was then diluted to 50 ml. with water and 0·45 gm. of sodium chloride was added to render the solution more nearly isotonic with blood. The solution was filtered if there had been much precipitation and was then used for the biological test. Guinea pigs weighing 400–600 gm. were used and anaesthetized with urethane, using a dose of 0·7 ml. per 100 gm. of a 25 p.c. solution. The guinea pigs were arranged for artificial respiration and the thorax was opened in order to observe the heart. The injection was made by means of a motor-driven syringe giving a constant rate of injection of 0·5 ml. per minute and the needle was usually inserted into the jugular vein.

TABLE 1.

Apocynaceae.

Plant	Part of plant tested	Equivalent wt. of dried plant per kilo of body wt. to produce toxic effect gm.	Remarks
Alstonia constricta (F. Muell)	Bark	1·7	Heart slowed and finally stopped
Alstonia scholaris R.Br.	Bark	15·0	Non toxic
Alyxia magnifolia	Leaves and stems	12·6	Non toxic
Alyxia spicata	Leaves and stems	10·6	Non toxic
Alyxia ruscifolia	Leaves	11·3	Non toxic
Carissa ovata R.Br.	Leaves and stems	3·0	Typical cardiac glycoside action
Cerbera dilatata	Kernels	0·35	Typical cardiac glycoside action
Cerbera floribunda	Kernels	0·31	Typical cardiac glycoside action
Cerbera manghas	Kernels (of fresh fruit)	0·20	Typical cardiac glycoside action
Ervatamia orientalis R.Br.	Leaves	13·2	Non toxic
Lochnera rosea (Reichb.)	Leaves and stems	16·8	Non toxic
Ochrosia elliptica (Labill.)	Leaves	14·8	Non toxic
Parsonsia eucalyptophylla (F. Muell.)	Leaves	14·5	Non toxic
Parsonsia straminea (R.Br.)	Leaves and stems	6·0	Heart normal then suddenly stopped
Parsonsia latifolia	Leaves and stems	18·0	Non toxic
Thevetia nereifolia	Leaves	3·2	Typical cardiac glycoside action
Wrightia millgar	Wood	14·5	Non toxic

RESULTS.

Tables 1 and 2 show the results obtained with plants of the Apocynaceae and Asclepiadaceae respectively. In each case the figures given are the weight of dried plant corresponding to the amount of the extract injected and represent the average of experiments on two to four guinea pigs. Where no toxic symptoms were observed the injection was continued for at least half an hour or until an equivalent of over 10 gm. of plant material had been injected.

TABLE 2.

Asclepiadaceae.

Plant	Part of plant tested	Equivalent wt. of dried plant per kilo of body wt. to produce toxic effect gm.	Remarks
Gomphocarpus fruticosus (L.) *Asclepia fruticosa* R.Br.	Leaves and stems	1·6	Typical cardiac glycoside action
Cryptostegia grandiflora R.Br.	Leaves and stems	0·4	Extremely toxic cardiac glycoside action
Hoya australis R.Br.	Leaves and stems	15·0	Non toxic
Hoya australis	Fresh leaves and stems	18·5	Non toxic
Hoya nicholsonia	Stems	9·0	Heart slowed, slight fibrillation before death
Marsdenia rostrata R.Br.	Bark	6·4	Heart slowed and stopped, no fibrillation, etc.
Secamone elliptica R.Br.	Leaves and Stems	16·3	Non toxic
Tylophora grandiflora	Leaves and Stems	0·96	Heart stopped suddenly (insufficient sample for repetition)

It will be seen that a typical cardiac glycoside action is seen with approximately 0·3 gm. of *Cerbera sp.* and with about the equivalent of 3·0 gm. of *Carissa ovata* or *Thevetia nereifolia,* the latter being in fact well known as a source of thevetin. Of the Asclepiadaceae both *Gomphocarpus fruticosus* and *Cryptostegia grandiflora* showed cardiac glycoside toxicity, and the latter has been shown to contain cryptograndosides (Aebi and Reichstein, 1950). Two other species of Asclepias (physocarpa and curassavica) are already known to contain cardiac glycosides (Cornforth, 1950) and hence have not been included in this survey. It has been reported that *Hoya australis* is toxic to the heart but this was not observed in our experiments. The inclusion of the fresh material was, in fact, an effort to make sure that the drying process had not caused a loss of the toxic material expected. Work is proceeding in these laboratories on the isolation and pharmacological examination of glycosides from *Gomphocarpus fruticosa* and the three *Cerbera* species listed above.

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SUMMARY.

Plants of the *Apocynaceae* and *Asclepiadaceae* families obtained from Queensland have been tested for cardiac activity by observing the effect on the exposed heart of a guinea pig when extracts of the plants are injected intravenously.

Of the *Apocynaceae* family, the *Cerbera spp.*, *Carissa ovata* and *Thevetia nereifolia* showed typical cardiac glycoside action, and of the *Asclepiadaceae*, *Gomphocarpus fruticosus* (R.Br.) and *Cryptostigia grandiflora* (R.Br.) were the only two to show a typical cardiac glycoside activity.

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THE RESPONSE OF CAPILLARY ENDOTHELIUM TO CHANGES IN INTRAVASCULAR PRESSURE, AS SEEN IN THE RABBIT'S EAR CHAMBER

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During the last hundred years, many authors have noted two facts about capillary endothelial behaviour: (1) that sometimes the smooth outline of the capillary wall is distorted at intervals by the inward bulging of endothelial nuclei, without change of the external diameter; and (2) that there may be local or widespread constriction of the capillary lumen, with folding or shrinking of the wall. General agreement as to the cause or mechanism of these phenomena has not yet been reached and it is the aim of this paper to attempt to clarify the situation as it is met with in the ear-chamber preparation.

Most of the earlier studies were on amphibian capillary endothelium, in various preparations of frog membranes. These have been repeatedly and extensively reviewed, and will not be further considered in this paper (*vide* Vimtrup, 1922; Krogh, 1929; Midsuno, 1930; Lutz, Fulton and Akers, 1950). Capillary behaviour has also been studied in mammalian preparations, for instance by Midsuno (1930) (mesentery and tongue of frog, mesentery of rabbits, guinea-pigs, rats and mice); Rogers (1932) (mesentery of rabbit), (1934) (omentum of cat); Fields (1935) (frog membranes, subcutaneous tissues and mesentery of rat); Chambers and Zweifach (1944) (mesenteries of rats and dogs). Observations made on nail-fold capillaries (Moog and Ambrosius, 1922; Nesterow, 1925; Pelzer and Redisch, 1944) cannot accurately disclose the exact behaviour of the walls of these vessels, and have not contributed much to this subject. More recently two other mammalian preparations have been used, namely the cheek pouch of the American rodent, the Golden Hamster (Lutz, Fulton and Akers, 1950) and the web of the bat's wing (Nicoll and Webb, 1946, 1948). These permit examination of the small vessels at high magnification, as does the Zweifach-Chambers preparation of the rat meso-appendix.

A very suitable preparation for the microscopic study of small mammalian vessels is the transparent rabbit ear-chamber, first described by Sandison in 1924, and since then adapted and modified by many workers for many purposes. Some authors have recorded observations on the effect of a sympathetic stimulation on the vessels in these chambers, but interpretation of the findings has varied.

Beecher (1936, a, b) studied preformed-tissue chambers, and noted an instant swelling of the capillary endothelial nuclei when the animal was frightened or hurt; he also found

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that the injection of adrenalin subcutaneously near the central artery of the ear produced a similar response. Sanders, Ebert and Florey (1940), using thin-tissue chambers, about 6 weeks after vascularization, evoked swelling of the endothelial cells by stimulating the cervical sympathetic trunk. They assumed that in chambers of that age nerve-fibres would have grown into the observation area. van Dobben-Broekema and Dirken (1950), using preformed-tissue chambers, state that their photographs "clearly showed that the smallest vessels also constrict vigorously on stimulation of the sympathetic." However, the long exposures (20–30 seconds) which they used, must make this conclusion open to doubt. Harris and Marvin (1927) used cedar-wood oil to render the skin of the rabbit's ear transparent, and thus were, in effect, observing "pre-formed tissue," although the low magnifications employed would not have permitted clear differentiation between muscularized and true capillary vessels. They reported that after arrest of the circulation by occluding the external carotid artery and its branches, after ligation of the opposite common carotid, stimulation of the sympathetic trunk caused the small vessels (including the capillaries) to contract.

In opposition to these workers, Levinson and Essex (1943), using the thin-tissue type of chamber, about two weeks after vascularization, found that on sympathetic stimulation there was marked arteriolar constriction, but could see no evidence of independent capillary constriction. They considered that the disappearance of the lumina of the capillaries was secondary to the arteriolar change. The intravenous injection of adrenalin caused variable but essentially similar results.

Clark and Clark (1943), summing up nearly 20 years of experience with ear-chambers of all types, state that, in mammals, changes in the endothelial nuclei are passive only. Their view is strongly supported by those workers who have studied small vessels in other preparations. For example, Chambers (1946) ascribed the nuclear bulgings to the release of tension on the endothelial cell by contraction of a muscular cell outside. He considered that when the forces keeping the endothelial cell in its normally expanded and flattened form are reduced, the cell tends to round out, and thus permits the nucleus to bulge into the vessel. Nicoll and Webb (1946) did not believe that Rouget cells have any influence on capillary diameter, and added that changes in the endothelium will not suffice to explain changes in the lumen until the pressure can be measured in each case.

Clark, Clark and Williams (1934, 1938) demonstrated nerve-fibres by supra-vital methylene blue in newly-formed tissues in the ear-chamber (in one instance less than four weeks after insertion of the chamber). Robertson (1951, b) has also seen them in a similar preparation of an ear-chamber about 2 months old. No other reports on this method have been found in the literature. An attempt was made, in conjunction with the present experiments, to develop this technique with a view to relating regional vascular behaviour to the presence or absence of nerve-fibres, but reliable results were not obtained. Owing to the thickness of the preparation phase-contrast microscopy was of no assistance in searching for nerve-fibres.

It is the aim of this paper to discuss the forces acting on the capillary wall, and the effect on it of reduction of intravascular pressure.

Methods.

Chambers. Those used were of the Florey-Pullinger design, modified slightly after the model described by Robertson (1951, a). The base was made from perspex sheet ⅛ inch (3·15 mm.) thick, turned to a disc 30 mm. in diameter, with a central table 1 mm. high, 5 mm. in diameter at the top and 6 mm. at the base. The use of ⅛-inch sheet preserved the polished surfaces. In the earlier model made from thicker sheet, the base was machined, and this gave rise to objectionable diffraction effects. The other modification is the use of three thin (0·050 mm.) perspex buffers, made from sheet cut into spears and cemented with shellac in spirit to the underside of the mica window in the lid. The spears were placed so that their tips just overlapped the edge of the central table. They were much more convenient to handle than the former buffers which were cemented directly to the table itself (Fig. 1).

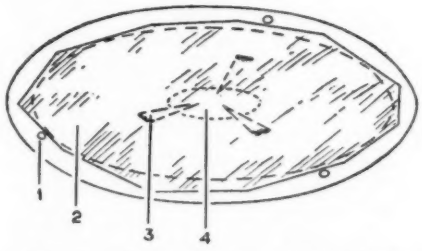

Fig. 1. Sketch (not to scale) of undersurface of lid, showing (1) perspex ring, with three bolt-holes, (2) thin mica window, (3) perspex sheet spears cemented to mica, (4) dotted line showing location of table-top when assembled.

The chambers were inserted with the base to the dorsal aspect of the ear, the method being much as described by Robertson, except that it was found possible to simplify the aseptic technique. The operator's hands were washed with soap and water, rinsed in "Centrimide" and dried on a sterile towel. No gloves were used. The skin of the ear was prepared by shaving with electric clippers, and painted with 1 p.c. iodine in 70 p.c. alcohol, dispensing with the former prolonged soaking in acriflavine. 200,000 units of Procaine Penicillin were given intramuscularly after the operation. With this technique 10 consecutive chambers were inserted without loss by infection.

Sympathectomy. This was performed after the fully vascularized chamber had been studied, generally about 6 weeks after insertion. Following the accepted view that such measures are adequate to denervate the distal half of the ear, the cervical sympathetic ganglion was excised, and approximately 1 cm. of the dorsal and ventral auricular nerves resected (Feldberg, 1926); Lewin and Schilf, 1927; Grant, Bland and Camp, 1932; White, Okelberry and Whitelaw, 1936; Le Compte, 1942; van Dobben-Broekema and Dirken, 1950). No attempt was made to approach the stellate ganglion. It was found most convenient to approach the cervical sympathetic trunk from the lateral aspect, through an extension downwards and forwards of the incision made to expose the auricular nerves. The wound was closed with Michel clips, and chemotherapy given as above. Six operations were performed without untoward effects, and five of these animals were used in the experiments after histological examination had confirmed the removal of the ganglion.

Stimulation. The sympathetic trunk was prepared through a lateral incision, and stimulated faradically by a silver electrode applied to the nerve as needed; the indifferent electrode was inserted into the animal's rectum. The source of stimuli was a Harvard Inductorium driven by a thyrotron circuit at a frequency of about 15/second.

Staining for nerves. Four rabbits were used, some more than once, the chamber ages ranging from 2 to 12 months. These animals were not used in the main experiments. Both intravenous and intra-arterial methods, as described by Clark, Clark and Williams (1934)

were employed. Methylene blue (Grübler n. Ehrlich) was made up as a 1/200 solution in physiological saline. Direct application of the dye was not feasible because of the danger of injuring the tissues when raising the lid of the chamber.

Observations. These were conducted on the animals, both when un-anaesthetized and when under Nembutal (average dose 60 mg. in 1 ml. intravenously) with supplementary ether by inhalation.

The optical equipment was that described by Robertson (1951).

Photography. These preparations were difficult to photograph, but eventually the following was found to give best results. A pale green filter was employed, with exposures of 1/25 to 1/5 seconds made on Ilford Pan-F film, depending on the objective used. Films were developed for 5 minutes at 70° F. in Ilford Developer ID-14, diluted 1:2 with water. Prints were made on Ilford B-5 paper developed in a strong caustic process developer. Some pictures were also made using a 100-Joule electronic flash lamp, but it was very difficult to control the illumination so provided, and while good exposures were occasionally obtained, the method proved too unreliable for general use.

RESULTS.

Observations were made on the effect of changes in intravascular pressure on capillary diameter. Despite the warning of Harris and Marvin (1927), collateral circulation was not found to interfere greatly with the results, and the circulation in most of the vessels on the table was arrested during:

(1) Compression of the central artery of the ear between finger and thumb. (Similar pressure applied alongside the central artery had no effect; light pressure applied directly over it, and sufficient only to occlude accompanying veins, also had no effect.) Ten rabbits were used, and five of them were studied at intervals up to two weeks after sympathectomy.

(2) Occlusion of the common carotid artery by a loop of thread. (Four rabbits were used, eight weeks after insertion of chamber.)

(3) General arterial constriction following sympathetic stimulation. (The same four rabbits were used as for (2).)

The changes during circulatory arrest varied from chamber to chamber, and from area to area, but reduction of intravascular pressure by any one of the three methods gave a similar result in a given chamber. Stimulation of the sympathetic did not affect capillaries which were unaffected by arterial occlusion, and sympathectomy did not alter the response to compression of the central artery of the ear.

The effects could be divided into two groups, circulatory and vascular changes and tissue changes.

Circulatory and vascular.

Arterioles. On arterial occlusion the flow was arrested in most arterioles and generally, but not always, slowed in the others. On the whole these vessels decreased only slightly in diameter but in some areas where there was marked

shift of tissues (see below) they were compressed, along with the venules and capillaries.

On sympathetic stimulation, similar changes were observed, except that in two cases the outline of a previously unaffected vessel became irregular, presumably from the contraction of muscular elements of the wall; the neighbouring capillaries, however, showed no change.

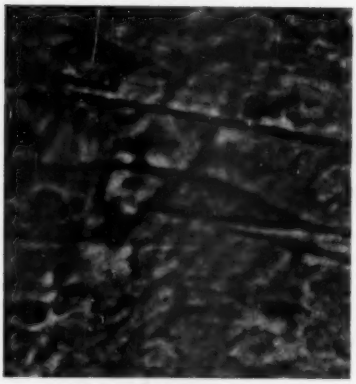

Fig. 2.

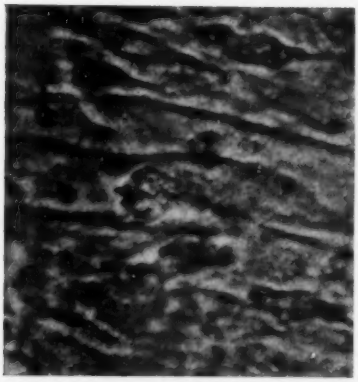

Fig. 3.

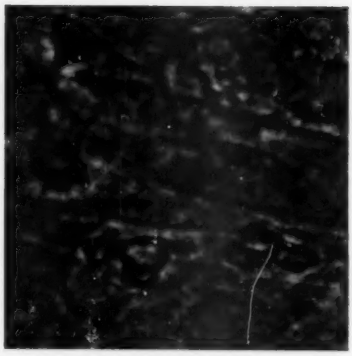

Fig. 4.

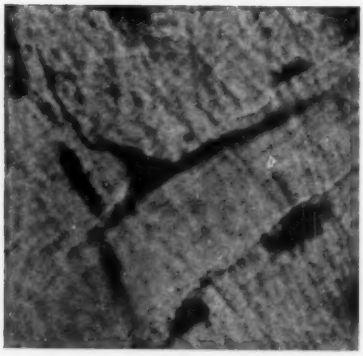

Fig. 5.

Figs. 2–4 show an endothelial cell in a chamber 3 weeks old, while the central artery of the ear is (2) not compressed, (3) slightly compressed, (4) completely occluded.

Fig. 5 shows a branching nerve-fibre, associated with a triangular nucleus, and closely related to a capillary. Stained macrophages are also present. Supravital methylene blue preparation.

Venules. After arterial occlusion the blood in these vessels continued to drain away for a short time, but the flow then ceased, except in those areas still supplied by an arteriole. Decrease in venular diameter was variable, and usually small, but after release of arterial occlusion a reflux of blood often occurred, the venules filling from the venous end. After a short period of oscillation the circulation regained its normal direction.

On sympathetic stimulation similar changes occurred.

Lymphatic vessels. These were present only in the older chambers. The usual finding was that the cells suspended within the lumen moved slightly towards the edge of the table. In one instance in which such a vessel decreased in calibre as a result of tissue shift, the endothelial cells bulged into the lumen exactly as in a blood capillary.

Blood capillaries. The findings after arterial occlusion varied. In some chambers no change was detected in any region; the capillaries remained open, containing motionless blood, and showing no appreciable change in diameter. In most chambers, however, a region or regions, amounting sometimes to as much as $\frac{1}{3}$ or $\frac{1}{2}$ of the table area, could be seen, extending in for a variable distance from the edge of the table, where with a drop in intravascular pressure, the capillaries immediately closed. The endothelial cells rounded up and bulged into the lumen, which became tortuous. The contained blood-cells were forced out in either or both directions, and in less than one second the capillary pattern virtually disappeared. Similar changes occurred at the growing tips of capillaries, and on the course of newly-formed vessels, as well as in mature vessels in chambers where the pattern had become stable. By carefully varying the degree of arterial compression these changes could be modified at will, from a slight degree of intrusion of the nuclei, without appreciable change in the external diameter of the vessel, to complete obliteration of the lumen, and the distortion of any cells trapped within. (See Figs. 2, 3, 4.) After release of compression the capillaries filled again, mostly from the arterial end, but usually in some measure by venous reflux.

The changes on sympathetic stimulation were exactly as above, except that the process was rather slower, and become fully developed only after about 10 seconds. Changes were not seen in those capillaries which had remained unaffected on arterial occlusion.

Tissue changes.

Chambers of this design permitted the inspection of the preformed tissues surrounding the table, although these had rather poor optical properties.

Floating cells. Between the tissues and the lid of the chamber there was a fluid layer in which were suspended quite large numbers of macrophages, some of which adhered to the mica, while others were floating and were seen to move

back and forth synchronously with the arterial pulse. Particular attention was paid to those cells lying just above the thin tissue, at or just inside the edge of the table. When the intravascular pressure was reduced they generally moved slightly towards the edge of the table, but this was not an invariable finding. The floating cells beyond the table area often streamed about rapidly as the circulation was occluded.

Vessels. On arterial occlusion, flow in the small arteries slowed or stopped and their forms changed, becoming narrower and a little more tortuous. It was often seen that a vessel approaching the central table remained patent and contained cells until the table itself was reached, where the lumen was obliterated.

As far as optical conditions would permit, no evidence was found for contraction of capillaries in preformed tissue, either on arterial compression or sympathetic stimulation.

Staining for nerves. Since the technique could not be developed to give significant or dependable results, and was found to carry considerable risk of damage to the tissues, it was not employed on the chambers used in the main experiment. By the intravenous route, 4 or 5 ml. of the methylene blue solution were introduced into the vessels of the ear while its base was compressed for 2 or 3 minutes. Some of the dye reached some of the vessels on the table, where perivascular macrophages stained deeply. In the preformed tissue surrounding the table, some nerve-fibres were stained in relation to the large vessels, but staining was restricted to a narrow region near these, beyond which the nerves could not be traced. A similar situation was met with by Woollard, Weddell and Harpman (1940). In one chamber, 12 months old, a nerve-fibre was demonstrated in relation to a venule. It appeared as a branching structure, associated with a triangular nucleus, presumably of a Schwann cell. A photomicrograph of this is reproduced as Fig. 5.

Although the intravenous route gave the better staining, the solution did not enter all the vessels on the table, and staining was consequently patchy. By the intra-arterial route, the solution could be much more evenly distributed, the ear being virtually perfused with it for one or two minutes, but the subsequent vascular spasm led to anoxia which inhibited the staining. In two cases thrombosis of the artery led to necrosis of the tissues of the chamber.

DISCUSSION.

The precise anatomy of the termination of the autonomic fibres in relation to capillary vessels is still in dispute, mainly as a result of the conflicting evidence afforded by the two methods of study. Methylene blue techniques show a loosely ramifying network casually related to the capillary pattern, possibly being thus aligned by tissue stresses during development (Nicoll and Webb,

1946). Silver impregnation methods, on the other hand, show a much finer and more widespread and intimate system of fibres, with intercalated cells. The opponents of this technique maintain that silver is not sufficiently specific, and that the so-called "terminal reticulum" is of connective-tissue origin. The whole field has been well reviewed by Busch (1929), Michels (1935), Nonidez (1936), Weddell *et al.* (1940), Fulton and Lutz (1942), Nelemans (1948), Feindel, Allison and Weddell (1948), and more briefly by Boyd (1952).

Insofar as the present investigations are concerned with the nervous control of the capillaries, the details of anatomy are, however, less important than the broad question of whether there were or were not nerve-fibres in the thin-tissue chambers. It was therefore regrettable that the supravital staining technique could not be developed to provide reliable evidence of their presence or absence. It is, however, reasonable to assume with Sanders, Ebert and Florey (1940), and with other workers who have used this preparation for similar studies, that nerve-fibres will have grown into chambers six to eight weeks old, as they have been demonstrated in chambers of this age on a few occasions (Clark, Clark and Williams, 1934; Robertson, 1951b). Indirect evidence for their presence was found in the two cases where stimulation of the cervical sympathetic trunk caused irregular contraction of the arterioles.

The tone or contractility of the capillaries in the thin-tissue chambers was not influenced by the sympathetic system, since these observations showed that sympathectomy did not affect the results of arterial occlusion, and sympathetic stimulation evoked only those changes which were produced by arterial occlusion.

The protrusion of endothelial nuclei into the capillary lumen must therefore be regarded as a passive effect; indeed, it is difficult to explain such rapidly reversible changes of shape in any other way. The extra-capillary (Rouget) cells were not involved in these changes, their contours remaining practically unaltered.

The explanation of the shutting of the capillaries and the swelling of the endothelial nuclei must therefore be sought in terms of the mechanical forces acting on the capillary wall.

The inherent elasticity of the endothelial cells. Clark and Clark (1943) state that under normal conditions, the diameter of a capillary is slowly adapted to changes of flow, over the course of days. Chambers and Zweifach (1946) also hold this view. It is reasonable to assume that over the relatively short periods of the observations recorded, and in the absence of trauma, the "tone" of a capillary will remain constant.

Interfacial tension. Burton (1951) and Nichol *et al.* (1951) have advanced a hypothesis that there exists a certain critical intravascular pressure, below which the vessel will shut. This is explained in terms of the elasticity of the wall, and the presence of an interfacial tension which is attributed to the incomplete wet-

ting of endothelium by plasma. The following observation, however, which was made in this laboratory in the course of other experiments, must cast some doubt on the existence of such a tension. A few bubbles of air had been inadvertently injected into the vessels in the preformed tissue, and the air-blood interface was seen to be steeply parabolic, indicating no marked lack of wetting of the endothelial wall.

Hydrostatic pressure difference between contents and surroundings. This difference will, of course, be affected by changes in the general pressure obtaining within the chamber; but in the absence of venous obstruction the latter must remain essentially constant. The flow of suspended particles, as described above, indicated that compensatory volume changes were taking place in the veins of the preformed tissues as the arteries decreased in size. Increase in diameter was seen in some veins; this only followed arterial compression and was not caused by compression of the veins accompanying the central artery of the ear.

Mechanical stresses in the tissues. These were probably the most important forces concerned, being generated by the changes in volume and shape of the elastic vessels of the preformed tissue, and transmitted to the vessels on the table by the fibrous tissue in which these were embedded. In the presence of such forces the small vessels were sheared, the capillaries being most affected, while the more robust arterioles and venules were influenced less. It has been seen, for example, that a venule was sheared and obliterated, together with all capillaries lying to one side of it, while those on the other side were not changed. The possibility also exists that some descent of the lid may have taken place, as was mentioned by Sandison (1932) and Wilson (1936); but in a chamber with the buffers properly aligned this is not at all likely to have occurred. The slight movement of cells towards the edge of the table may possibly have been due to such a descent, but more probably the flow of fluid was responsible.

The state of a capillary will be determined at any given time by the "tone" and elasticity of its wall, the mechanical stresses in the surrounding tissues, and the difference in pressure between its contents and the surrounding fluid. When the intravascular pressure was reduced by occluding the main arterial supply, either by direct pressure or by stimulation of the sympathetic, the vessels in the thin-tissue chamber were subjected to forces which could not be assessed. In a given vascular network, where flow was arrested and the intravascular pressure consequently equal throughout, some parts of the network showed constriction of vessels, while others did not change. It follows, therefore, that the ear-chamber technique is unreliable for quantitative experimental work involving the measurement of vascular pressures and diameters.

SUMMARY.

In a series of 10 rabbits, the capillaries in certain areas of thin-tissue ear-chambers were constricted to varying degrees following compression of the central artery of the ear, and responded instantly to changes in intravascular pressure. Sympathectomy did not influence this response.

Four rabbits were studied 8 weeks after insertion of chambers, and in any one, essentially the same response of capillary constriction and endothelial nuclear prominence was elicited by compression of the central artery of the ear, compression of the common carotid, and stimulation of the cervical sympathetic trunk.

An attempt was made to develop the technique of supravital methylene blue staining, so that the location of nerve-fibres could be determined. The method was found to be unreliable and carried the risk of injury to the tissues; it was therefore not pursued.

It is concluded that the capillary behaviour is passive only, and is determined by the elasticity of the endothelial wall, the intravascular pressure, and the mechanical stresses in the surrounding tissues.

Since, in the ear-chamber, stresses which cannot be estimated are set up in the preformed tissue by movement of large elastic vessels, and transmitted by the fibrous tissue, this technique will not provide accurate quantitative data where vascular diameters and pressures are to be measured.

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